Hypermethylation of the hMLH1 Gene Promoter in Human Gastric Cancers with Microsatellite Instability

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ABSTRACT

Human gastric carcinoma shows a higher prevalence of microsatellite instability (MSI) than does any other type of sporadic human cancer. The reasons for this high frequency of MSI are not yet known. In contrast to endometrial and colorectal carcinoma, mutations of the DNA mismatch repair (MMR) genes hMLH1 or hMSH2 have not been described in gastric carcinoma. However, hypermethylation of the hMLH1 MMR gene promoter is quite common in MSI-positive endometrial and colorectal cancers. This hypermethylation has been associated with hMLH1 transcriptional blockade, which is reversible with demethylation, suggesting that an epigenetic mechanism underlies hMLH1 gene inactivation and MMR deficiency. Therefore, we studied the prevalence of hMLH1 promoter hypermethylation in a total of 65 gastric tumors: 18 with frequent MSI (MSI-H), 8 with infrequent MSI (MSI-L), and 39 that were MSI negative. We found a striking association between hMLH1 promoter hypermethylation and MSI; of 18 MSI-H tumors, 14 (77.8%) showed hypermethylation, whereas 6 of 8 MSI-L tumors (75%) were hypermethylated at hMLH1. In contrast, only 1 of 39 (2.6%) MSI-negative tumors demonstrated hMLH1 hypermethylation (P < 0.0001 for MSI-H or MSI-L versus MSI-negative). Moreover, hypermethylated cancers demonstrated diminished expression of hMLH1 protein by both immunohistochemistry and Western blotting, whereas nonhypermethylated tumors expressed abundant hMLH1 protein. These data indicate that hypermethylation of hMLH1 is strongly associated with MSI in gastric cancers and suggest an epigenetic mechanism by which defective MMR occurs in this group of cancers.

INTRODUCTION

Gastric cancer is the fourth leading cancer worldwide and the leading cancer in several countries. Its etiology is unknown, but strong associations exist with several dietary or environmental factors and with gastric mucosal infection by the bacterium Helicobacter pylori (1, 2).

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MSI comprises length mutations in tandem oligonucleotide repeats that occur in a large subset of human tumors (3–6). This type of mutation is believed to be caused by altered DNA MMR (7–10). Among human sporadic tumors, gastric carcinoma possesses the highest prevalence of MSI, with up to 33% of cases manifesting this abnormality (5, 11–14). The underlying cause of MSI in gastric tumors is unknown: mutations in the four major MMR genes, i.e., hMSH2, hMLH1, hPM2, and hPMS2, have not yet been described, although secondary mutations in the MMR genes hMSH3 and hMSH6 occur in gastric tumors already manifesting MSI (15, 16). In MSI-positive sporadic colorectal and endometrial tumors, hypermethylation of the hMLH1 gene promoter is extremely frequent and often accompanied by down-regulation of hMLH1 gene expression (17–20). Furthermore, reversal of methylation by treatment of cells with 5-aza-2’-deoxycytidine results in reexpression of hMLH1 protein and restoration of MMR function in MMR-deficient colorectal cancer cells (18). Taken together, these findings suggest a possible mechanism by which failure of MMR occurs in these tumors.

To evaluate the possible involvement of hMLH1 hypermethylation in gastric carcinogenesis, we investigated a series of 65 sporadic gastric tumors: 18 with frequent MSI (MSI-H), 8 with infrequent MSI (MSI-L), and 39 without MSI.

PATIENTS AND METHODS

Patients. A total of 65 sporadic gastric adenocarcinomas and 65 matching blood or normal gastric control tissues were studied. DNA samples provided by coauthors originated from patients in the People’s Republic of China, Japan, South Korea, Italy, and the United States. Tissue specimens were stored at −180°C until study. When necessary, additional genomic DNA was obtained from primary tumors, using a previously described DNA extraction technique (21).

MSI. A subset of samples that were not previously characterized for MSI were tested at loci D2S123, BAT25, BAT26, Mfd15, DSS346, D2S119, D11S904, and D2S147 using methods described previously (5, 22). All other samples had been tested previously for MSI at various combinations of microsatellite loci. Chinese tumors were tested at IFNA, D9S171, D9S26, D9S162, D9S165, and D9S162; Korean samples were tested at D2S119, D2S123, D2S147, D10S197, and D11S904; Japanese samples were tested at D2S111, D4S40, D4S409, D5S265, D7S522, D11S940, MYH7, TP53, D18S69, and D21S1436; and samples from the University of Virginia were tested at 20 anonymous microsatellite loci (available on request). Tumors were characterized as MSI-H if they manifested instability at two or more loci (or >30% of loci if more than five loci were tested), MSI-L if unstable at only one locus (or <30% of loci if more than five loci were tested), and MSI-negative if they showed no instability at any loci.

MSP. DNA methylation patterns in the hMLH1 promoter were determined by methylation-specific PCR, as described previously (23). MSP distinguishes

<ref>hMLH1</ref>
unmethylated from hypermethylated alleles in a given gene based on sequence changes produced after bisulfite treatment of DNA, which converts unmethylated, but not methylated, cytosines to uracil, and subsequent PCR using primers specific to either methylated or unmethylated DNA. Briefly, 1 μg of genomic DNA was denatured by treatment with NaOH and modified by sodium bisulfite. In cases where only a limited quantity of DNA was available, as little as 50 ng of genomic DNA were used. DNA samples were then purified using Wizard DNA purification resin (Promega), again treated with NaOH, precipitated with ethanol, and resuspended in water. PCR was then performed using the primer pairs described below under the following conditions: the PCR mix contained 1× PCR buffer (16.6 mM ammonium sulfate, 67 mM Tris (pH 8.8), 6.7 mM MgCl₂, and 10 mM 2-mercaptoethanol), deoxynucleotide triphosphates (each at 1.25 mM), primers (300 ng each per reaction), and bisulfite-modified DNA (50 ng) in a final volume of 50 μl. Reactions were hot-started at 95°C for 5 min before the addition of 1.25 units of Taq polymerase (BRL). Amplification was carried out in a Hybaid OmniGene temperature cycler (Hybaid, Middlesex, United Kingdom) for 35 cycles (30 s at 95°C, 30 s at 59°C, then 30 s at 72°C, followed by a final 4-min extension at 72°C). Control PCRs lacking genomic DNA were performed for each set of reactions. Ten μl of each PCR reaction product were directly loaded onto nondenaturing 6% polyacrylamide gels, stained with ethidium bromide, and visualized under UV illumination.

Primer sequences of hMLH1 for the unmethylated reaction were 5'-TTT TGA TGT AGA TGT TTT ATT AGG GTT GT-3' (sense) and 5'-ACC ACC TCA TCA TAA CCA ACC ACA-3' (antisense), whereas for the methylated reaction they were 5'-AGC TAC AGC TTA TAT TAT GTT GTG CTC-3' (sense) and 5'-CAT CAT CAT AAC CAT CCG CGA-3' (antisense). PCR conditions were as described above for MSP, with an annealing temperature of 59°C. SW48 DNA, which is hypermethylated at the hMLH1 locus (20), was used as a positive control for hypermethylated hMLH1 alleles. DNA from normal lymphocytes was used as negative control for hypermethylated hMLH1.

**Immunohistochemistry.** Frozen tissue was thawed on ice, fixed in 10% formalin, and embedded in paraffin blocks. Five-μm sections were mounted on glass slides. Paraffin-embedded tissues were deparaffinized with xylene for 30 min and dehydrated using graded ethanols. Antigen retrieval was performed using a heat-induced epitope retrieval method (24). Immunoperoxidase staining using diaminobenzidine as chromogen was performed with the TechMate 1000 automatic staining system (Ventana; BioTek Solutions, Tucson, AZ). Mouse monoclonal antibody to the hMLH1 gene product was used at 1:300 dilution (PharMingen, San Diego, CA). Staining of tumor cells was evaluated using a heat-induced epitope retrieval method (24). Immunoperoxidase stainings were performed using primers amplifying the four loci D3S1298, D3S1561, D3S1611, and D3S1277 and delimited within a 1-cM interval bordered by markers D3S1298 and D3S1561. D3S1611 is actually located within an intron of hMLH1 (25). PCRs were performed using primers amplifying the four loci described above (Research Genetics, Huntsville, AL). LOH was defined as a shift in allele:allele signal ratio in tumor DNA of at least 50% relative to homologous normal control DNA.

**Statistical Correlations.** Analyses were performed using Statview 4.5 and superANOVA software for the Macintosh (SAS Institute Inc., Cary, NC). Two-by-two contingency analyses were performed using a two-tailed Fisher’s Exact test because some numerical values were less than five.

**RESULTS**

MSL. MSI status was already known on 51 samples. The remaining 14 tumors were analyzed for MSI as described above. Of these 14, one was MSI-high and two were MSI-low. When added to the total, 18 tumors were MSI-H (at least two of five loci or >30% of loci positive), 8 tumors were MSI-L (only one of five or <30% of loci was positive), and 39 cases were MSI negative (no positive loci).

**Methylation Analysis.** Results of hMLH1 promoter methylation assays versus MSI studies in 65 gastric cancer patients are summarized in Table 1 and Fig. 1. Hypermethylation of the hMLH1 promoter occurred in 14 of 18 MSI-H cases (77.8%) and 6 of 8 MSI-L cases (75%). Taking these two subgroups together, 20 of 26 cases

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**Table 1. Microsatellite instability, hMLH1 hypermethylation, and hMLH1 expression in gastric cancer**

<table>
<thead>
<tr>
<th>Sample no.</th>
<th>MSI status</th>
<th>Methylation</th>
<th>Western</th>
<th>Immunohistochemistry</th>
<th>LOH* (locus)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sh9</td>
<td>High</td>
<td>Methylated</td>
<td>Decreased</td>
<td>Decreased</td>
<td>DJS1277</td>
</tr>
<tr>
<td>G56,G63</td>
<td>High</td>
<td>Methylated</td>
<td>Decreased</td>
<td>ND</td>
<td>DJS1298</td>
</tr>
<tr>
<td>G106</td>
<td>High</td>
<td>Methylated</td>
<td>Decreased</td>
<td>ND</td>
<td>DJS1611</td>
</tr>
<tr>
<td>G119</td>
<td>High</td>
<td>Methylated</td>
<td>Increased</td>
<td>ND</td>
<td>DJS1298</td>
</tr>
<tr>
<td>Y9</td>
<td>High</td>
<td>Methylated</td>
<td>ND</td>
<td>Decreased</td>
<td>DJS1298</td>
</tr>
<tr>
<td>G16</td>
<td>High</td>
<td>Methylated</td>
<td>ND</td>
<td>ND</td>
<td>DJS1611</td>
</tr>
<tr>
<td>M112</td>
<td>High</td>
<td>Methylated</td>
<td>ND</td>
<td>ND</td>
<td>DJS1298</td>
</tr>
<tr>
<td>G39-41, G43, T15, G48</td>
<td>High</td>
<td>Methylated</td>
<td>ND</td>
<td>ND</td>
<td>DJS1298</td>
</tr>
<tr>
<td>G37-38, T22, G42</td>
<td>High</td>
<td>Unmethylated</td>
<td>ND</td>
<td>ND</td>
<td>DJS1611, DJS1298</td>
</tr>
<tr>
<td>Sh7</td>
<td>Low</td>
<td>Methylated</td>
<td>Decreased</td>
<td>Decreased</td>
<td>DJS1298</td>
</tr>
<tr>
<td>G99, M103, M135</td>
<td>Low</td>
<td>Methylated</td>
<td>ND</td>
<td>Decreased</td>
<td>ND</td>
</tr>
<tr>
<td>Y3</td>
<td>Low</td>
<td>Methylated</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>G1</td>
<td>Low</td>
<td>Methylated</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>M104</td>
<td>Low</td>
<td>Unmethylated</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>Y7</td>
<td>Low</td>
<td>Unmethylated</td>
<td>ND</td>
<td>Normal</td>
<td>ND</td>
</tr>
<tr>
<td>Ghs4</td>
<td>Negative</td>
<td>Methylated</td>
<td>Normal</td>
<td>Decreased</td>
<td>ND</td>
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<tr>
<td>JG7</td>
<td>Negative</td>
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<td>Normal</td>
<td>Increased</td>
<td>Normal</td>
</tr>
<tr>
<td>JG9</td>
<td>Negative</td>
<td>Unmethylated</td>
<td>ND</td>
<td>Normal</td>
<td>ND</td>
</tr>
<tr>
<td>Y2, Y4, Y6, Y8</td>
<td>Negative</td>
<td>Unmethylated</td>
<td>ND</td>
<td>Normal</td>
<td>ND</td>
</tr>
</tbody>
</table>

* LOH indicates loci showing allelic deletion (if positive).  
  † ND, not done.
The presence of PCR product in lanes marked 

* indicates hypermethylated. Water (H2O) serves as negative control for contaminating normal cells.

In seven of eight cases showing MSI and hMLH1 hypermethylation, Western blots revealed hMLH1 protein levels substantially lower in tumor than in matching normal control tissue (Fig. 4). One of these eight tumors (G119) displayed increased hMLH1 expression. When compared with matching normal control tissues, the two nonhypermethylated MSI-negative tumors subjected to Western blotting (JG9 and JG7) revealed unchanged protein expression in one case and increased protein expression in the second. Four cases (Sh7, Sh9, Sh4, and JG9) had expression of hMLH1 evaluated by both immunohistochemistry and Western blotting. In three of these cases, Western analysis corroborated immunohistochemical findings; hypermethylated tumors with MSI (Sh7 and Sh9) showed decreased hMLH1 expression in both analyses, whereas the unmethylated tumor without MSI (JG9) expressed hMLH1 by both methods. The single hypermethylated, MSI-negative case (Sh4) showed conflicting results, with decreased expression of hMLH1 in tumor tissue by immunohistochemistry but strong expression by Western analysis. Data are summarized in Table 1.

**DISCUSSION**

The above data show that hMLH1 promoter hypermethylation occurs at a very high frequency in sporadic gastric carcinomas exhibiting MSI but is rare in MSI-negative gastric cancers. Furthermore, the majority of gastric carcinomas with hMLH1 promoter hypermethylation exhibit decreased hMLH1 protein expression. This evidence suggests that hMLH1 promoter hypermethylation is associated with hMLH1 transcriptional inactivation and MMR deficiency in a large proportion of MSI-positive gastric carcinomas.

The present gastric carcinoma data are consistent with previous reports of hMLH1 hypermethylation in colorectal and endometrial cancers manifesting MSI (17–20, 26) and support the hypothesis that hypermethylation of the hMLH1 promoter is the most prevalent mech-
anism of MMR deficiency in sporadic human tumors. Although evidence connecting hypermethylation with gene inactivation has been largely associative, several studies suggest a direct causative link between promoter hypermethylation and decreased RNA expression. In one such study, cell lines from non-small cell lung carcinoma and head and neck squamous cell carcinoma were analyzed for methylation of the tumor suppressor gene p16 (27). Methylation of a CpG upstream of p16 was associated with complete transcriptional blockade, which was reversible upon treatment with 5-deoxyazacytidine, a demethylating agent (27). Even more relevant to the present data was a recent article showing a direct mechanistic connection between hMLH1 promoter hypermethylation and DNA MMR deficiency in MMR-deficient SW48 and RKO colorectal cancer cell lines (18). In the latter study, demethylation using 5-deoxyazacytidine led to restoration of hMLH1 protein expression and restitution of MMR proficiency (18). The theory that hMLH1 hypermethylation is important is particularly appealing in view of the rarity of MMR gene mutations in MSI-positive gastric cancers, despite the high rate of MSI in sporadic gastric tumors (5, 11–14).

The maintenance of MMR proficiency in the setting of partial hypermethylation suggests that hypermethylation of both alleles, or “complete” hypermethylation, of hMLH1 is required to produce the MSI phenotype. In the MMR-deficient cell lines SW48 and RKO, which are devoid of contaminating normal cells, only methylated hMLH1 PCR product is evident (18). Moreover, 5-deoxyazacytidine treatment of SW48 and RKO cells restores MMR proficiency, although this treatment achieves only partial demethylation (i.e., posttreatment methylation-specific PCR reveals both methylated and unmethylated products). In addition, the MMR-proficient colorectal cancer cell line HT29 contains both hypermethylated and unmethylated hMLH1 alleles (18), again suggesting that biallelic hypermethylation is required for MMR inactivation. Finally, in another study, an hMLH1 coding region polymorphism was demonstrated at codon 219 in Vaco5 and Vaco432 cell lines (26). After 5-azacytidine demethylation treatment, induction of transcripts bearing both maternal and paternal polymorphisms was detected in cDNA amplified from either cell line (26). This latter study clearly demonstrated biallelic inactivation of hMLH1 by hypermethylation. Finally, in the present study, an unmethylated PCR product was evident in cases with LOH and hypermethylation; and greatly reduced or absent hMLH1 protein expression was observed in cases with hypermethylation. We pre-

![Fig. 3. Immunohistochemical analyses of hMLH1 levels in primary gastric cancer. Low power (A) and high power (B) views of normal gastric mucosa expressing abundant hMLH1 protein in nuclei are shown. Low power (C) and high power (D) views of gastric cancer with MSI and hypermethylation of hMLH1, showing absence of detectable hMLH1 protein in neoplastic nuclei but its abundance in surrounding normal nuclei, are shown. Low power (E) and high power (F) views of gastric cancer negative for MSI and hypermethylation of hMLH1, showing hMLH1 protein in both normal and neoplastic cells, are shown.](https://cancerres.aacrjournals.org)

![Fig. 4. Western blotting analysis of hMLH1 expression in hypermethylated and unmethylated primary gastric cancers. The hMLH1 protein band is indicated. In MSI-H and MSI-L cases with hypermethylation (Sh9, G56, G63, Sh7, G99, M103, and M135), a substantial decrease in or complete absence of hMLH1 protein is noted in tumors (T) relative to matching normal control tissues (N). In unmethylated samples (JG7 and JG9), expression of hMLH1 protein in tumor tissue is unchanged or increased compared with matching normal controls.](https://cancerres.aacrjournals.org)
some that in our tumors lacking LOH, hypermethylation affected both alleles of hMLH1. These data support the hypothesis that biallelic inactivation of hMLH1 is required in order for MMR deficiency to occur.

One-half the patients with MSI and hMLH1 promoter methylation tested for LOH did display LOH at loci near the hMLH1 gene on chromosome 3p21.3. To our knowledge, frequent LOH at these loci has not been reported previously in gastric cancers. In these tumors, we hypothesize that hypermethylation of the remaining allele constitutes the “second hit” that results in MSI. However, it remains unclear whether LOH at hMLH1 predisposes toward hypermethylation of the remaining allele.

Our data lend additional support to the thesis that hMLH1 hypermethylation is a unifying feature in tumors associated with HNPPC kindreds. Gastric and endometrial cancers are associated with HNPPC at higher rates than in the normal population (28–30). In tumor types that rarely show the classic MMR-deficient phenotype and that are rare in HNPPC kindreds, such as breast and lung carcinomas or gliomas, hMLH1 is not hypermethylated (17). However, hMLH1 promoter hypermethylation occurs in the majority of sporadic colorectal, endometrial, and gastric carcinomas showing MSI (14, 15).

Thus, future studies of tumor frequencies in HNPPC kindreds may reveal additional tumor types in which hMLH1 promoter hypermethylation plays a key role.

Although consistent with hypermethylation rates in colorectal and endometrial cancer (17–20), our data showing hypermethylation in 76.5% of MSI-H gastric tumors may actually represent a conservative estimate; of 10 MSI-H tumors with DNA <1 μg per MSP assay, only 6 were hypermethylated. In contrast, where DNA quantities of 1 μg or greater were available, eight of eight MSI-H gastric cancers showed hypermethylation. Furthermore, to our knowledge, coding region mutations in the major MMR genes have rarely, if ever, been described in gastric cancer. Therefore, we predict that hMLH1 hypermethylation is more frequent in gastric than in colorectal or endometrial cancers, in which MMR gene mutations have been described. Other possible mechanisms underlying MMR deficiency in the MSI-positive gastric cancers lacking hMLH1 hypermethylation include: (a) hypermethylation of other known MMR genes; (b) point mutations in known MMR genes; or (c) mutations of as yet unidentified MMR genes.

Finally, another interesting feature of the present data was the occurrence of hypermethylation in five of six MSI-L tumors. Traditionally, MSI-L tumors are grouped together with MSI-negative tumors, due to evidence supporting highly similar biologies for these two subgroups (31, 32). However, much of the published literature does not describe data for MSI-L tumors separately from MSI-negative cases, and a distinct biology may exist for MSI-L lesions. For example, serrated colorectal adenomas are more often MSI-L than MSI-H (33). We speculate that in MSI-L gastric cancers, hypermethylation represents a recent event: hMLH1 hypermethylation initiates MSI, leaving a temporal window during which some tumors have not yet accumulated large numbers of microsatellite alterations. This theory may also explain the single case in our series (Sh4) in which hMLH1 hypermethylation occurred without MSI. It is also consistent with diminished hMLH1 expression in our MSI-L tumors. The low frequency of such cases suggests that this temporal window is brief. Furthermore, the partially hypermethylated cell line HT29 manifests MMR proficieny (18). Thus, early hypermethylation may be partial or monoallelic, with full-blown (high) MSI only becoming manifest at the later stage of complete (or biallelic) hypermethylation.

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