**hMLH1 Promoter Methylation and Lack of hMLH1 Expression in Sporadic Gastric Carcinomas with High-Frequency Microsatellite Instability**

Suet Yi Leung, 2,3 Siu Tsan Yuen, 2 Lap Ping Chung, Kent Man Chu, Annie S. Y. Chan, and Joana C. I. Ho

Departments of Pathology [S. Y. L., S. T. Y., L. P. C., A. S. Y. C., J. C. I. H.] and Surgery [K. M. C.], The University of Hong Kong, Queen Mary Hospital, Hong Kong

**ABSTRACT**

Mutation of DNA mismatch repair genes has rarely been documented in sporadic gastric carcinoma with microsatellite instability (MSI). In sporadic colorectal carcinoma, hMLH1 promoter methylation associated with protein loss is found in the majority of high-frequency MSI cases. We investigated a series of 35 sporadic gastric carcinomas stratified into high-frequency MSI (MSI-H), low-frequency MSI (MSI-L) and microsatellite stable (MSS) groups and found that hypermethylation of the CpG island in the hMLH1 promoter region was present in 100% of MSI-H sporadic gastric carcinomas. In 90% of cases, there was an associated complete loss of hMLH1 protein, as detected by immunohistochemistry, and a markedly lowered hMLH1 mRNA level. This loss of hMLH1 protein occurred in the MSI-H invasive tumor but not in the adjacent carcinoma-in situ or dysplastic components that were MSS. The MSI-L and MSS forms of gastric carcinoma all showed predominately unmethylated hMLH1 promoter, positive hMLH1 protein and high hMLH1 mRNA level. On the other hand, hMSH2 protein was expressed in all of the tumors irrespective of the MSI status. Our results suggest that high-frequency MSI in sporadic gastric cancer is mostly due to epigenetic inactivation of hMLH1 in association with promoter methylation, and the loss of hMLH1 protein is a significant event in the development of invasive tumor.

**INTRODUCTION**

MSI has been found in 15–39% of sporadic gastric carcinomas worldwide (1–9). Although MSI is present in a high proportion of colorectal carcinomas in the young (10, 11), the MSI type of gastric carcinomas are mostly seen in the elderly. Gastric carcinomas with a high-frequency of MSI are associated with specific clinicopathological characteristics including (a) an antral location; (b) intestinal type differentiation; (c) more abundant lymphoid infiltrate; (d) less lymph node metastasis; and (e) better survival rate (4–6, 8, 9). This clustering suggests that these cases may constitute a group with a common and specific pathway for carcinogenesis. MSI is present in the majority of tumors developing in hereditary nonpolyposis colon cancer syndrome and germline DNA MMR genes mutation can be detected in more than 60% of these individuals (12–16). However, the cause of MSI in sporadic tumors is less clear. Somatic mutation in one of the MMR genes has been found in up to 26% of sporadic MSI colorectal carcinomas (17–22) and 22% of sporadic MSI endometrial carcinomas (23–25). Germline MMR gene mutation on the other hand, is mostly limited to young patients among the sporadic colorectal carcinoma cases (10, 11).

Immunohistochemical staining has proven to be a useful adjunct in examining the DNA MMR system. Studies on tumors with MSI and germline MMR gene mutations have shown that immunohistochemistry can predict the presence of a defective DNA MMR component. The loss of the corresponding protein is seen in nearly all of the tumors with germline MMR gene mutation, except for the occasional case with missense mutation (21, 26, 27). Interestingly, in a recent study (28), 90% of sporadic high-frequency MSI colorectal carcinomas were found to have lost the hMLH1 protein.

Ablation of methylation of the promoter has been known to cause transcriptional silencing of some important tumor suppressor genes, such as p16 (29–31), E-cadherin (32), and the von Hippel Lindau (VHL) gene (33), and this has been implicated in the carcinogenic process in many cancers (see review, Ref. 34). Loss of the hMLH1 mRNA or protein was initially found in two colorectal cancer cell lines and in a few sporadic colorectal carcinomas associated with aberrant methylation of the hMLH1 promoter (35). More recently, promoter methylation associated with loss of the hMLH1 protein was found in some MMR defective colorectal and endometrial cancer cell lines in which mutation in the 4 MMR genes, including hMLH2, hMLH1, hMSM2 and hPM2, was absent. Treatment of these cell lines with 5-azacytidine restored the expression of hMLH1 protein and MMR capability (36, 37). hMLH1 promoter methylation and protein loss were also noted in high proportion of sporadic MSI colorectal carcinoma samples (36, 38). On the other hand, the promoter of hMSH2 was rarely methylated. These data suggest that aberrant methylation of the hMLH1 promoter is potentially a very important mechanism in the deactivation of the MMR system in colorectal carcinoma, but little is known about this process and its role in gastric cancer.

We used monoclonal antibodies to screen for the loss of hMSH2 and hMLH1 proteins in a series of MSI-H, MSI-L and MSS gastric cancers and investigated the underlying mechanisms for the loss of the respective MMR protein.

**MATERIALS AND METHODS**

**Tumors.** Thirty-five primary gastric carcinomas were selected from an ongoing study of 130 cases that had been tested for microsatellite instability using at least 6 of 8 loci, including both dinucleotide repeats (Dp53, D18S57, D2S123, D5S346) and polyadenine tracts (BAT26, BAT40, BAT-RFl). The results from a major proportion of these cases have been reported previously (9). All of the 11 MSI-H, all of the 5 MSI-L, and 19 randomly selected MSS gastric carcinomas among the 130 cases were evaluated for the relationship of MSI to MMR protein expression, promoter methylation, and MMR gene mRNA level. Cases were referred to as MSI-H if there were more than 40% unstable loci, MSI-L if less than 40% unstable loci, and MSS if no unstable loci (9, 39, 40). The methods used were as described previously (9). The microsatellite loci alterations in the MSI cases are listed in Table 1.

**Immunohistochemistry.** Immunostaining for hMSH2 and hMLH1 were performed in all of the cases using the standard streptavidin-biotin peroxidase complex method as described previously (27). Six-micron thick sections of 10% neutral buffered formalin-fixed, paraffin-embedded tumor tissue were incubated for 1 h at 37°C with monoclonal antibodies against the NH2-terminal fragment (clone GB12; dilution 1:20; Oncogene Research Products, Cambridge, MA) and COOH-terminal fragment of hMLH2 (clone FE11; dilution 1:200; Oncogene Research Products, Cambridge, MA). For hMLH1, sections were incubated at 37°C for 1 h using a monoclonal antibody (clone G168–15; dilution 1:10; PharMingen, San Diego, CA). Microwave pretreatment at 95°C for 30 min in citrate buffer (pH 6.0) was performed after deparaffinization. For negative control, the primary antibodies were replaced with mouse IgG (Da-
haplotypes. The staining results were examined and graded without knowledge of the MSI status. Normal tissues were taken as internal positive control, and cases were graded only if there was positive nuclear staining in the regenerative mucosal cells and lymphoid cells in the germinal center. The stains were graded: (a) negative when there was complete absence of staining in the tumor cell nuclei; and (b) positive when there was positive staining in the tumor cell nuclei.

**hMLH1 Promoter Methylation.** The methods used were similar to those of Kane et al. (35) with some modification. pBluescript II KS phagemid DNA (4.4 pg) were added to 250 ng of genomic DNA in a 30-μl volume of restriction endonuclease buffer. The pBluescript II DNA serve as internal control for cleavage by HpaII and MspI because the CpG sites in the recognition sequences of these enzymes are not cystosine-methylated and can be cleaved to completion by both enzymes. The reactions contained either no enzyme, 25 units of HpaII, or 20 units of MspI for 12 h at 37°C.

The cleavage of the hMLH1 promoter region was analyzed by a pair of primers similar to Kane et al. (35), amplifying nucleotides −670 to −67 of hMLH1. Four HpaII sites in positions −567, −527, −347, and −341 were included in this region. Twenty ng of each digest was analyzed by PCR in 25-μl reactions containing 10 m M Tris-HCl (pH 8.3), 50 m M KCl, 1 m M MgCl2, 0.2 m M dNTP, 0.5 units of Taq, and 0.1 m M each primers. PCR was performed for one cycle of 95°C for 5 min, followed by 35 cycles of 94°C for 30 s, 55°C for 30 s, and 72°C for 30 s, followed by one cycle of 72°C for 7 min. The PCR products were analyzed by agarose gel electrophoresis. Analysis of DNA methylation was performed in the absence of knowledge of the hMLH1 expression status in all of the experiments.

Cleavage of the control pBluescript II DNA was analyzed as above with the M13 universal primers, flanking a 209-bp region with two HpaII sites.

**hMLH1 Expression by RT-PCR.** Total RNA was extracted from frozen blocks using the standard guanidium thiocyanate method. Guided by the frozen sections, the tumor areas were dissected out from the blocks for RNA extraction. The RNA was then subjected to electrophoresis on 1% agarose gel to ensure the quality. For reverse transcription, cDNA was generated in a 20 μl reaction containing 0.2 m M dNTP, 2.5 m M MgCl2, 1 unit Taq DNA polymerase, buffer containing 50 m M Tris-HCl (pH 8.3), 50 m M KCl supplied by the manufacturer, and 5 μl cDNA. Twenty-five cycles of 45 s at 94°C, 45 s at 60°C, and 45 s at 72°C were carried out in an automatic thermal cycler. The products were visualized by gel electrophoresis and ethidium bromide staining. The cDNA dilution was chosen in which the amount of target and competitor ampiclons were similar in intensity as determined by the GDS8000 Complete Gel Documentation and Analysis System (UV Products, Cambridge, United Kingdom) based on the volume intensity of the bands. After optimal tumor cDNA dilution was selected, the reaction was duplicated to ensure reproducible results.

The normalized cDNA was subjected to PCR using a pair of primers flanking nucleotides 471-1104 of the hMLH1 cDNA (forward primer, 5'-CAACTAGGACCGAGGAGAA; reverse primer, 5'-CGAGGTCAGACTTGTTGGGA). The PCR condition was similar to that described above with minor modifications. Thirty cycles of amplification were performed using 60 μl reaction volume containing 3 m M MgCl2, and the resultant band was visualized by gel electrophoresis and ethidium bromide staining. The cycle number was chosen to ensure that the amplification was not in the plateau phase. Serial dilutions of the tumor cDNA were added to the PCR reaction. The PCR reaction was carried out in a 25-μl reaction containing 0.4 μl of each forward and reverse β-actin primer, 0.2 m M dNTP, 2.5 m M MgCl2, 1 unit Taq DNA polymerase, buffer containing 50 m M Tris-HCl (pH 8.3), and 5 μl cDNA. Twenty-five cycles of 45 s at 94°C, 45 s at 60°C, and 45 s at 72°C were carried out in an automatic thermal cycler. The products were visualized by gel electrophoresis and ethidium bromide staining. The cDNA dilution was chosen in which the amount of target and competitor ampiclons were similar in intensity as determined by the GDS8000 Complete Gel Documentation and Analysis System (UV Products, Cambridge, United Kingdom) based on the volume intensity of the bands. After optimal tumor cDNA dilution was selected, the reaction was duplicated to ensure reproducible results.

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**RESULTS**

The results of MMR protein expression, hMLH1 promoter methylation, and hMLH1 gene expression are summarized in Table 2.

**Immunohistochemistry.** Both hMSH2 and hMLH1 staining of the non-neoplastic tissue were consistent with those reported previously (21, 26, 42, 43), with strong positive nuclear staining of cells in the
and MSS cases have unmethylated genes sensitive to HpaII digestion but sensitive to digestion by MspI, consistent with methylation of all of the four HpaII sites (Fig. 2). Ten of these 11 cases had complete loss of the hMLH1 protein, and the remaining case gave a weak positive staining.

The DNA isolated from all of the 5 MSI-L and 19 MSS cases were sensitive to digestion by both HpaII and MspI (Fig. 2). In four of the MSS cases, a small amount of HpaII-resistant DNA was seen despite replenishment with excess restriction endonuclease. This suggests the presence of a small population of tumor cells with hMLH1 promoter methylation or possibly that one allele of the hMLH1 was methylated. In all of the MSI-L and MSS cases, the MLH1 protein was expressed in the tumor. In all cases, the unmethylated internal control DNA was completely digested by HpaII and MspI.

The association of MSI-H with hMLH1 promoter methylation and hMLH1 protein loss was statistically highly significant using the Fisher’s exact test ($P < 0.00001$).

hMLH1 mRNA Expression by RT-PCR. The mRNA expression in the 11 MSI-H, 5 MSI-L and 14 MSS cases was assessed by RT-PCR; representative results are shown in Fig. 3. The mean level of hMLH1 mRNA in cases with MSI-H and hMLH1 promoter methylation was significantly lower than the MSI-L ($P = 0.009$) and MSS cases ($P = 0.0001$) using the Mann-Whitney $U$ test (Fig. 4 and Table 2). The hMLH1 mRNA level was also significantly lower in the tumors with no hMLH1 protein ($P < 0.0001$).

**DISCUSSION**

Our data document the importance of aberrant methylation of the hMLH1 promoter in causing MSI-H in sporadic gastric cancer. Aberrant hMLH1 promoter methylation was present in 100% of the MSI-H cases. This is associated with a lowered level of mRNA and a total absence of the hMLH1 protein in more than 90% of cases, supporting the former as the cause of transcriptional silencing of this gene. The remaining weak mRNA expression in the MSI-H, hMLH1 promoter methylated, and protein-negative cases is most probably derived from the admixed normal cells within the tumor. Although we cannot exclude the possibility of mutation being the underlying cause for the loss of hMLH1 protein and MSI phenotype in some of these cases with promoter methylation, this possibility even if it exists, is estimated to account for only a small proportion of cases. Somatic mutation in one of the MMR genes has been detected in at most 26% of sporadic MSI cancers in various organs (17–25). In sporadic gastric cancer, data on MMR gene mutation is scarce, and thus far only five
somatic mutations in the MMR genes have been reported (44, 45). On the other hand, methylation of the CpG island in the promoter is known to cause transcriptional silencing of many tumor suppressor genes, and de-methylation of the hMLH1 promoter has been shown to restore the hMLH1 protein expression and MMR capacity in colorectal cancer cell lines (36, 37).

Our findings are that the loss of the hMLH1 protein is a significant event operating in tumor progression rather than initiation in gastric cancer. The preservation of the hMLH1 protein in intestinal metaplasia, dysplasia, and carcinoma in situ phases, and its complete absence in the whole invasive tumor associated with onset of MSI suggests that there is rapid clonal expansion and invasive tumor formation once the hMLH1 protein is lost. This may be achieved by accelerated mutation of important growth regulatory genes such as transforming growth factor β type II receptor or Bax. An analogy can be drawn with the colorectal adenoma-carcinoma sequence where MSI has been found to play a role in tumor progression rather than in the initiation phase. MSI is only rarely found in adenomas and there is a significant increase of MSI in benign adenoma with areas of malignancy and in cancer (46–50).

Interestingly, none of the MSI-L gastric carcinomas showed loss of either hMSH2 or hMLH1 proteins, a result similar to the MSS cases. This supports the previous observation that the MSI-H cases had specific clinicopathological features (4–6, 8, 9), and the mechanism for the MMR defect is probably different from the MSI-L cases. Similarly, MSI-L colorectal carcinomas were indistinguishable from the MSS counterparts in clinicopathological features, and they had normal hMSH2 and hMLH1 protein expression (28). It is possible that another component of the MMR system may be inactivated by yet unknown mechanisms in the MSI-L cancers.

A small amount of HpaII-resistant DNA was detected in four MSS gastric carcinomas with positive hMLH1 protein expression. Similar observations were noted in a few cases of MSS colorectal carcinomas (36, 38) and MSS endometrial carcinomas (51). These cases may represent the presence of a small population of tumor cells with methylation or hemimethylation. Also, one MSI-H gastric carcinoma showed prominent promoter methylation but still expressed the hMLH1 protein, and a similar case of MSI colorectal carcinoma was noted previously (36). It is likely that methylation of the CpG sites analyzed is important but not sufficient for gene inactivation. This is not surprising because the density of CpG island methylation in the 5’ regulatory region in general correlates with the degree of transcriptional repression in a gene. Further study to map the methylation status of CpG sites in a larger region and to correlate with protein expression may help to resolve these issues.

The cause of the deranged methylation remains poorly understood. Taken together with data from previous studies in which methylation of a variety of endogenous genes and exogenous retroviral gene sequences were seen more frequently in MMR-deficient colorectal cancers when compared with their MMR proficient counterparts (52, 53), MMR deficiency in sporadic gastric cancer may in fact be the result of a deranged methylation state in the individual. This aberrant methylation can cause cancer by inactivation of the hMLH1 promoter and lead to the mutator phenotype. Similarly, this mechanism can also inactivate other important growth regulatory genes. Moreover, this aberrant methylation status appears to be an actively maintained process in MSI-cancer cell lines; the hMLH1 expression can be turned on and off with 5-azacytidine exposure and washout (37). Further study to define the mechanism underlying this methylation process will be crucial in our understanding of this type of epigenetic event in carcinogenesis.

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