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Hypermethylation of the *hMLH1* Gene Promoter in Human Gastric Cancers with Microsatellite Instability[1]

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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).

MSI[6] 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*, *hPMS1*, 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*, *D5S346*, *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*, *D9S126*, *D9S162*, *D9S104*, *D9S165*, and *D9S163*; Korean samples were tested at *D2S119*, *D2S123*, *D2S147*, *D10S197*, and *D11S904*; Japanese samples were tested at *D2S111*, *D4S404*, *D5S409*, *D6S265*, *D7S522*, *D11S490*, *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

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[6] The abbreviations used are: MSI, microsatellite instability; MMR, mismatch repair; MSP, methylation-specific PCR; LOH, loss of heterozygosity; HNPCC, hereditary nonpolyposis colon cancer.

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 $MgCl_2$, 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 CTA CCC ACA-3′ (antisense), whereas for the methylated reaction they were 5′-ACG TAG ACG TTT TAT TAG GGT CGC-3′ (sense) and 5′-CCT CAT CGT AAC TAC CCG CG-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 xylenes 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 by M. E. and J. G. H. as present or absent in stained slides.

Western Blotting. Sections of frozen tissue were prepared in ice-cold RIPA buffer (1× Tris-buffered saline, 1% NP40, 0.5% sodium deoxycholate, and 0.1% SDS) at a concentration of 200 μg/ml, with 10 mg/ml phenylmethylsulfonyl fluoride in isopropranalol added at 10 μl/ml, with aprotinin (Sigma) added at 30 μl/ml, and with 100 mM sodium orthovanadate added at 10 μl/ml. Protein concentrations of soluble supernatants from each sample were measured by the Bio-Rad protein assay (Bio-Rad, Hercules, CA). One hundred μg of protein for each sample were loaded per lane on a 4–15% Tris-HCl polyacrylamide gel (Bio-Rad) and electrophoresed at 80 V for 1 h at room temperature. Equal loading was also verified by staining of parallel gels with Coumassie Blue. Proteins were then transferred to Trans-Blot transfer medium nitrocellulose membrane (Bio-Rad) by electroblotting. After blocking of nitrocellulose membranes in 5% nonfat dry milk in Tris-buffered saline at room temperature for 30 min, hMLH1 protein was detected by overnight incubation with a rabbit polyclonal antibody against a human peptide of hMLH1 (N-20; Santa Cruz Biotechnology, Santa Cruz, CA) at 1:500 dilution. This was followed by incubation with an anti-rabbit secondary antibody conjugated to horseradish peroxidase at room temperature for 1 h. Bands were visualized by ECL (enhanced chemiluminescence; Amersham, St. Louis, MO) and exposed to Kodak BioMax MR film for 20 s to 3 min.

LOH Assays. To determine whether the locus containing the *hMLH1* gene was heterozygously deleted in tumors, LOH was assayed. The gene for *hMLH1* resides on chromosome 3p21.3, within 1 cM of markers previously linked to cancer susceptibility in HNPCC kindreds. These markers are centered at loci *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 table contingency analyses were performed using a two-tailed Fisher's Exact test because some numerical values were less than five.

RESULTS

MSI. 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 hypermethylation 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

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

Sample no.	MSI status	Methylation	Western	Immunohisto-chemistry	LOH[a] (locus)
Sh9	High	Methylated	Decreased	Decreased	
G56,G63	High	Methylated	Decreased	ND[b]	*D3S1277*
G106	High	Methylated	Decreased	ND	
G119	High	Methylated	Increased	ND	*D3S1298*
Y5	High	Methylated	ND	Decreased	
G16	High	Methylated	ND	ND	*D3S1298*
M112	High	Methylated	ND	ND	*D3S1611*
G39-41, G43, T15, G48	High	Methylated	ND	ND	
G37-38, T22, G42	High	Unmethylated	ND	ND	
Sh7	Low	Methylated	Decreased	Decreased	*D3S1611*
G99, M103, M135	Low	Methylated	Decreased	ND	
Y3	Low	Methylated	ND	Decreased	
G1	Low	Methylated	ND	ND	*D3S1611, D3S1298*
M104	Low	Unmethylated	ND	ND	
Y7	Low	Unmethylated	ND	Normal	
Gsh4	Negative	Methylated	Normal	Decreased	
JG7	Negative	Unmethylated	Normal	ND	
JG9	Negative	Unmethylated	Increased	Normal	
Y2, Y4, Y6, Y8	Negative	Unmethylated	ND	Normal	
Sh1-3, Sh5-6, Sh8, Sh10-11, G2-5, G7-8, G13-15, G17, G20-25, G27, T13, Y1, Y9-13	Negative	Unmethylated	ND	ND	

[a] LOH indicates loci showing allelic deletion (if positive).
[b] ND, not done.

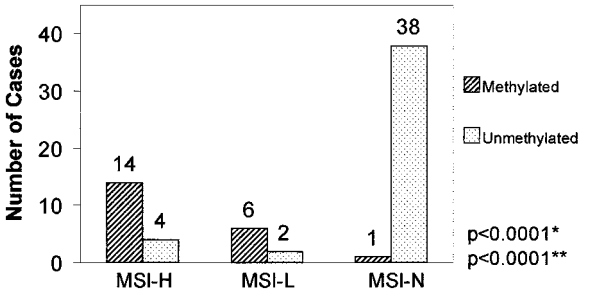

Fig. 1. hMLH1 promoter hypermethylation *versus* MSI status in gastric cancer. *MSI-H, MSI-L,* and *MSI-N* denote MSI-high, MSI-low and MSI-negative, respectively. *, $P < 0.0001$ for association of hypermethylation with MSI-H plus MSI-L *versus* MSI-N cases (Fisher's Exact test). **, $P < 0.0001$ for association of hypermethylation with MSI-H *versus* MSI-L plus MSI-N cases (Fisher's Exact test).

(76.9%) showing either low or high MSI were hypermethylated. In contrast, only 1 of 39 MSI-negative patients (2.6%) exhibited *hMLH1* promoter hypermethylation ($P < 0.0001$ for MSI-H plus MSI-L *versus* MSI-negative, Fisher's Exact test, two-tailed). Even when the MSI-L cases were considered separately from the MSI-H cases, both subgroups significantly differed from the MSI-negative group ($P < 0.0001$ for MSI-H *versus* MSI-negative *or* MSI-L *versus* MSI-negative, Fisher's Exact test, two-sided). Unmethylated signal was visible in all primary tumors, presumably as a result of normal cell contamination. Representative examples of methylation assay data are displayed in Fig. 2.

LOH. Of fourteen cancers demonstrating hypermethylation of *hMLH1* and available for testing, seven (50%) showed LOH at loci neighboring *hMLH1* on chromosome 3p. Specifically, LOH involved both *D3S1611* and *D3S1298* in one case, *D3S1611* alone in two cases, *D3S1298* alone in two cases, and *D3S1277* alone in two cases. Results are summarized in Table 1.

Immunohistochemistry. Four cases (Sh7, Sh9, Y3, and Y5) showing *hMLH1* hypermethylation and MSI were tested for hMLH1 protein expression using immunohistochemistry. All of these cases showed markedly diminished hMLH1 expression in tumor cell nuclei relative to normal cells in the same sections or in control normal mucosa. In addition, six MSI-negative samples (JG9, Y2, Y4, Y6, Y7, and Y8) lacking hypermethylation of *hMLH1* showed expression of hMLH1 protein in the nuclei of tumor and normal cells. Finally, the only tumor (Sh4) that was MSI negative and hypermethylated showed decreased expression of hMLH1 in tumor nuclei. Representative cases are displayed in Fig. 3, and data are summarized in Table 1.

Western Blotting. Western blotting for the detection of hMLH1 protein was performed on protein homogenates of gastric tissue in eight cases (Sh9, G56, G63, Sh7, G99, M103, M135, and G119) with *hMLH1* hypermethylation and either high or low MSI, as well as in two cases (JG9 and JG7) that were MSI negative and nonhypermethylated. 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-

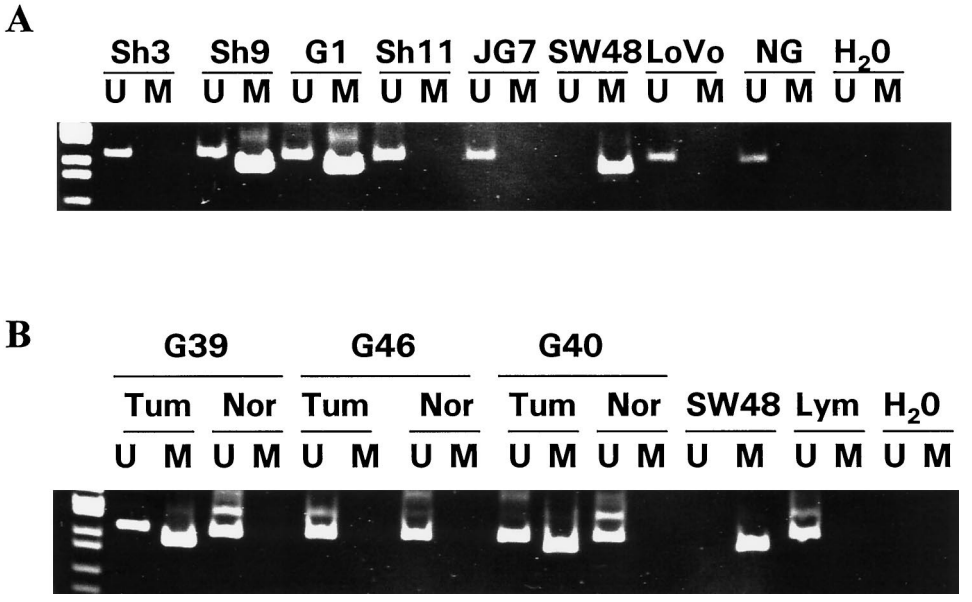

Fig. 2. Hypermethylation of *hMLH1* promoter region CpG island in primary human gastric carcinomas. The presence of PCR product in lanes marked *U* indicates unmethylated *hMLH1*; product in lanes marked *M* indicates hypermethylated *hMLH1*. Water (H_2O) serves as negative control for the PCR reaction (*A* and *B*). All primary tumors display weak unmethylated bands resulting from contaminating normal cells. *Left*, marker lane. *A*, MSI-negative (*Sh3, Sh11,* and *JG7*), MSI-low (*G1*), and MSI-high (*Sh9*) gastric cancers. Hypermethylated SW48 cells are positive controls, whereas unmatched normal gastric (*NG*) tissue and the unhypermethylated cell line LoVo are negative controls. *B*, MSI-high (*G39* and *G40*) and MSI-negative (*G46*) gastric cancers (*Tum*) with matching normal control stomach (*Nor*); also displayed are nonhypermethylated lymphocytes (*Lym*) and hypermethylated SW48 cells.

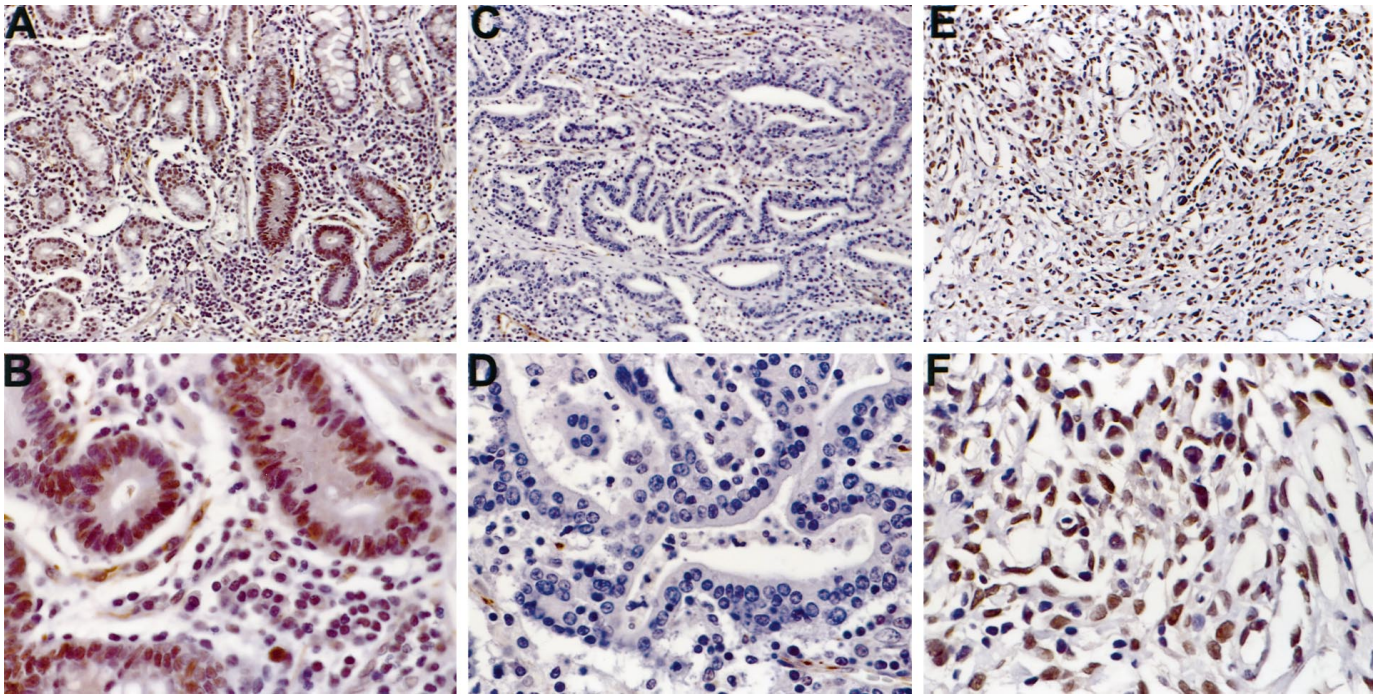

Fig. 3. Immunohistochemical analyses of hMLH1 levels in primary gastric cancer. Low power (*A*) and high power (*B*) views of normal gastric mucosa expressing abundant hMLH1 protein in nuclei are shown. Low power (*C*) and high power (*D*) views of gastric cancer with MSI and hypermethylation of *hMLH1*, 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.

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-

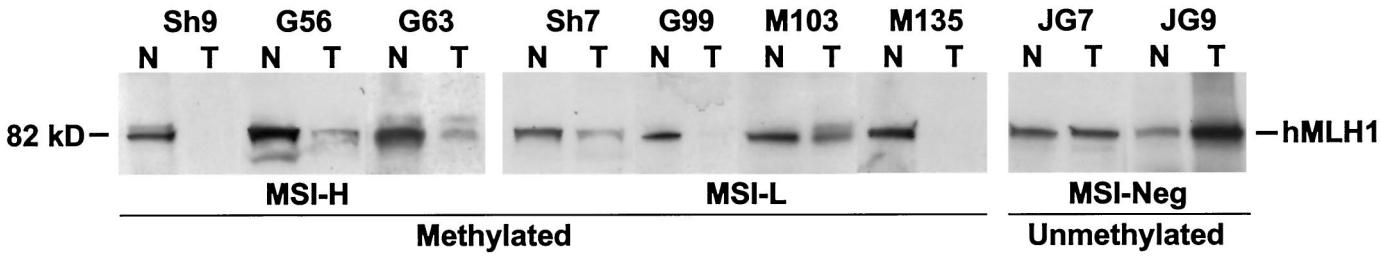

Fig. 4. Western blotting analysis of hMLH1 expression in hypermethylated and unmethylated primary gastric cancers. The hMLH1 protein band is indicated. In MSI-H and MSI-L cases with hypermethylation (*Sh9, G56, G63, Sh7, G99, M103,* and *M135*), a substantial decrease in or complete absence of hMLH1 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.

sume 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 HNPCC kindreds. Gastric and endometrial cancers are associated with HNPCC 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 HNPCC 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 HNPCC 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.[7] 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 tumors 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 tumors, 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 proficiency (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.

[7] M-G. Rhyu and J-Y. Lei, unpublished data.

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