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• GASTRIC CANCER •

Mutation and methylation of hMLH1 in gastric carcinomas with microsatellite instability

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Abstract

AIM: To appraise the correlation of mutation and methylation of hMSH1 with microsatellite instability (MSI) in gastric cancers.

METHODS: Mutation of hMLH1 was detected by Two-dimensional electrophoresis (Two-D) and DNA sequencing; Methylation of hMLH1 promoter was measured with methylation-specific PCR; MSI was analyzed by PCR-based methods.

RESULTS: Sixty-eight cases of sporadic gastric carcinoma were studied for mutation and methylation of hMLH1 promoter and MSI. Three mutations were found, two of them were caused by a single bp substitution and one was caused by a 2 bp substitution, which displayed similar Two-D band pattern. Methylation of hMLH1 promoter was detected in 11(16.2 %) gastric cancer. By using five MSI markers, MSI in at least one locus was detected in 17/68(25 %) of the tumors analyzed. Three hMLH1 mutations were all detected in MSI-H ($\geqslant$2 loci, n=8), but no mutation was found in MSI-L (only one locus, n=9) or MSS (tumor lacking MSI or stable, n=51). Methylation frequency of hMLH1 in MSI-H (87.5 %, 7/8) was significantly higher than that in MSI-L (11.1 %, 1/9) or MSS (5.9 %, 3/51) (P<0.01-0.001), but no difference was found between MSI-L and MSS (P>0.05).

CONCLUSION: Both mutation and methylation of hMLH1 are involved in the MSI pathway but not related to the LOH pathway in gastric carcinogenesis.

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INTRODUCTION

Our previous studies indicated that genetic instability may play an important role in gastric carcinogenesis[1]. There are at least two distinct genetic instabilities in gastric tumorigenesis: one is the chromosomal instability (or suppressor pathway) and the other is microsatellite instability (or MSI pathway). In the former, perhaps including tumors with low-frequency MSI (MSI-L) as well as microsatellite stable (MSS), accumulation of loss of tumor suppressor genes such as p53, Rb, APC, MCC and DCC play an important role in their carcinogenesis; whereas in the latter, consisting of a small subset of gastric cancer with high-frequency MSI (MSI-H), defective repair of mismatched bases results in an increased mutation rate at the nucleotide level, and the consequent widespread MSI[2-4].

Mismatch repair is required for the cell to accurately copy its genome during cellular proliferation. Deficiencies of this system result in mutation rates 100-fold greater than those observed in normal cells[5]. MSI is a hallmark of mismatch repair gene (MMR)-deficient cancers. MSI in tumors from patients with hereditary non-polyposis colorectal cancer (HNPCC) is caused by germline mutations in MMR genes, principally hMSH2 and hMLH1[6-10]. In contrast, somatic mutations in MMR genes are relatively rare in sporadic MSI+ colon cancers[9,11]. Rather, the majority of negative mutation, MSI+ cases involve hypermethylation of the hMLH1 promoter and subsequent lack of expression of hMLH1[12-16]. The details of the mechanisms of this epigenetic gene silencing remain to be elucidated in gastric cancer. The aim of this study was to define the mutation and methylation of hMLH1 in gastric carcinomas with MSI.

MATERIALS AND METHODS

Tissue samples

Sixty-eight cancer and corresponding normal tissues were obtained from surgically resected gastric carcinoma in our hospital. Each specimen was frozen immediately and stored at -80 ℃ until analyzed. A 5 μm section was cut from each tissue and stained with hematoxylin/eosin in order to ascertain whether the cancer cells in the tissues were predominant or not. Genomic DNA was isolated by standard proteinase-K digestion and phenol-chloroform extraction protocols. Of the 68 patients with gastric cancer, 45 were men and 23 were women with an age range of 30-76 years (a mean of 56.2 years at diagnosis). None of the patients included in the present series had a family history suggestive of HNPCC or had received chemotherapy or radiation therapy.

hMLH1 mutation analysis

PCR and heteroduplexing Primer pairs for long-chain and short-chain PCR and GC-clamped primers used were shown in Table 1[17]. PCR reactions were carried out in 50 μl reactions in thin-walled tubes in a Perkin-Elmer 2 400 thermocycler. A total of 200-400 ng of genomic DNA, varying concentrations of each primer, and the LA PCR kit (TaKaRa, Otsu, Shiga, Japan) were used for long PCR. Final concentrations of each LA PCR primer pair were as follows: hMLH1-4F and hMLH1-4R, 0.16 μM each; hMLH5-10F and hMLH5-10R, 0.125 μM each; hMLH11-13F and hMLH11-13R, 0.094 μM each; and hMLH14-19F and hMLH14-19R, 0.125 μM each. The reactions were carried out according to the manufacturer's instructions. In brief, the conditions were as follows: a hot start of 94 ℃ for 2 min, with the addition of Taq Pol in between,

followed by eight cycles of 98 ℃×20 sec, 69 ℃×1 min (with decrements of 0.5 ℃/cycle), and 68 ℃×12 min; six cycles of 96 ℃×20 sec, and 68 ℃×12 min; 16 cycles of 96 ℃×20 sec, and 68 ℃×12 min (with increments of 15 sec/cycle), and finally a chain extension of 72 ℃ for 10 min.

Table 1 Primer information for long and short PCR for HMLH1

A. Primer pairs for long-distance PCR

Exons 1-4
MLH1-4F GCG.GCT.AAG.CTA.CAG.CTG.AAG.GAA.GAA.CGT.GA[a]
MLH1-4R GGC.GAG.ACA.GGA.TTA.CTC.TGA.GAC.CTA.GGC.CC
Product size-10.8kb
Exons5-10
MLH5-10F GCG.CCC.CTT.GGG.ATT.AGT.ATC.TAT.CTC.TCT.ACT.GG
MLH5-10R GCG.CTC.ATC.TCT.TTC.AAA.GAG.GAG.AGC.CTG
Product size-10.5kb
Exons11-13
MLH11-13F CGG.CTT.TTT.CTC.CCC.CTC.CCA.CTA.TCT.AAG.G
MLH11-13R GGG.TTA.GTA.AAG.GAA.GAG.GAG.CTT.GCC.C
Product size-8.7kb
Exons14-19
MLH14-19F GGT.GCT.TTG.GTC.AAT.GAA.GTG.GGG.TTG.GTA.G
MLH14-19R GCG.CGC.GTA.TGT.TGG.TAC.ACT.TTG.TAT.ATC.ACA.C
Product size=10.5kb

B. Primer pairs for short PCR

Exon	Clamp[b]	Product size	T_m[c]	Primer sequence
1	5	258	64.13	GCA.CTT.CCG.TTG.AGC.ATC
	40			CCG.TTA.AGT.CGT.AGC.CCT
2	40	187	38.14	ATA.AAT.TAT.TTT.CTG.TTT
				CAT.CCT.GCT.ACT.TTG.AGG
3	40	237	32.22	GGA.AAA.TGA.GTA.ACA.TGA
	2			TGT.CAT.CAC.AGG.AGG.ATA
4	2	218	36.26	ACC.CAG.CAG.TGA.GTT.TT
	40			GCC.CAA.AAT.ACA.TTT.CAG
5	40	170	30.19	ATA.TTA.ATT.TGT.TAT.ATT
				CAA.TTT.ACT.CTC.CCA.TGT
6	40	228	35.58	TTT.CAA.GTA.CTT.CTA.TGA
				ACT.TTG.TAG.ACA.AAT.CTC
7		194	30.88	GAC.ATC.TAG.TGT.GTG.TTT
	40			CCC.CTT.TTT.TCT.TTT.CAT
8	5	213	42.21	GAC.AAT.AAA.TCC.TTG.TGT
	50			AAG.ATT.TTT.TTA.TAT.AGG
9	40	249	33.73	TTT.GAG.TTT.TGA.GTA.TTT
				TGG.GTG.TTT.CCT.GTG.AGT
10	50	240	41.47	CAC.CCC.TCA.GGA.CAG.TTT
				ACA.TCT.GTT.CCT.TGT.GAG
11.1	50	145	40.58	AGG.TAA.TTG.TTC.TCT.CTT
				GAA.GTG.AAC.TTC.ATG.CTT
11.2	40	224	60.81	TCC.CAA.GAA.TGT.GGA.TGT
	2			AAA.GGC.CCC.AGA.GAA.GTA
12.1	40	184	44.53	TTT.TTT.TTT.TTT.TAA.TAC.A
				AAT.CTG.TAC.GAA.CCA.TCT
12.2	8	366	53.23	TGG.AAG.TAG.TGA.TAA.GGT
	40			TGT.ACT.TTT.CCC.AAA.AGG
13	40	272	49.06	ATC.TGC.ACT.TCC.TTT.TCT
				AAA.ACC.TTG.GCA.GTT.GAG
14	45	235	48.94	TAC.TTA.CCT.GTT.TTT.TGG
	5			GTA.GTA.GCT.CTG.CTT.GTT
15	40	179	29.97	CAG.CTT.TTC.CTT.AAA.GTC
				CAG.TTG.AAA.TGT.CAG.AAG
16		261	47.56	CTT.GCT.CCT.TCA.TGT.TCT.TG
	40			AGA.AGT.ATA.AGA.ATG.GCT.GTC
17	40	199	47.01	ATT.ATT.TCT.TGT.TCC.CTT
				AAT.GCT.TAG.TAT.CTG.CCT
18	45	215	46.67	CCT.ATT.TTG.AGG.TAT.TGA.AT
				GCC.AGT.GTG.CAT.CAC.CA
19		282	43.43	TGT.TGG.GAT.GCA.AAC.AGG
	40			ATC.CCA.CAG.TGC.ATA.AAT

[a]Underlined nucleotides represented nucleotides added to modify the melting temperatures of the primers. [b]GClampsm are: 50clamp,CGC.CCG.CCG.CCG.CCC.GCC.GCG.CCC.CGC. GCC.CGT .CCC.GCC.GCC.CCC.GCC.CG; 45 GC clamp, CGC. CCG.CCG.CGC.CCC.CGC.CCC.GTC.CCG.CCG.CCC.CCG. CCC.GGC.CCG; 40 clamp, CGC.CCGCCG.CGC.CCC.GCG. CCC.GGC.CCG.CCG.CCC.CCG.CCC.G; 8 clamp, CGT.CCC. GC; 5 clamp, GCG. CG; 2 clamp,CG; [c]TM is given in %UF.

After checking and visualizing the long PCR products on a 0.8 % agarose gel, 1μl of the long PCR amplicons was used as template for subsequent extensive multiplex short PCR. The short PCR was performed in two multiplex groups of 11 and 10 amplicons, respectively. The final concentrations of each primer were shown in Table 2. The final concentrations of the PCR mix included 1×PCR buffer, 7 mM $MgCl_2$, and 0.25 mM each dNTP. Cycling conditions included a hot start of 3 min at 94 ℃ with the Taq Pol added after 2 min, followed by five cycles of 94 ℃×1 min, 52 ℃×45 sec (decrements of 1 ℃/cycle), and 72 ℃×1 min; 15 cycles of 94 ℃×1 min, 48 ℃×45 sec, and 72 ℃×1 min, 30 sec (with increments of 2 sec/cycle); 15 cycles of 94 ℃×1 min, 38 ℃×45 sec, and 72 ℃×1 min 30 sec. Each PCR reaction was teminated with a round of heteroduplexing: 72 ℃×10 min, 98 ℃×10 min, 45 ℃×30 min, and finally 37 ℃×30 min. Each tube reaction was directly mixed with 1/10 volume of 10×loading buffer, 6.5 μl of multiplex group I and 8.5 μl of multiplex group II were loaded onto a slab gel for size separation.

Table 2 Multiplex groups for short PCR

Multipex group I		Multipex group II	
Exon	Final concentration	Exon	Final concentration
11.1	0.375 μM	5	1.5 μM
15	0.5 μM	2	1.25 μM
12.1	0.375 μM	7	1.75 μM
17	0.5 μM	4	1.625 μM
8	0.375 μM	11.2	0.5 μM
18	1.25 μM	6	1.625 μM
14	1.25 μM	9	1.5 μM
3	0.625 μM	1	1.25 μM
10	0.625 μM	13	1.625 μM
16	1.0 μM	12.2	0.325 μM
19	1.75 μM		

Two-dimensional electrophoresis For two-dimensional electrophoresis, the DGGE instrument was from CBS Scientific Co. (Solana Beach, CA. Amplicons from each of the two multiplex reactions were mixed and subjected to electrophoresis in 0.5×TAE (Tris-Acetate EDTA). A 10 % polyacrylamide, 0.75 mm thick slab gel was used, the amplicons were fractionated at 140V for 7.5hr at 50 ℃. The separation pattern was detected by SYBR green staining and UV-transillumination of the slab gel. The 120-to 420-bp region in the middle of each lane was quickly cut out and applied to a 1mm thick DGGE gel.

The DGGE gel was prepared as a 1 mm thick slab gel with a 10-6.5 % reverse polyacrylamide gradient containing 25-70 % urea/formamide (UF) and 3-9 % glycerol gradients. Electrophoresis was carried out for 16 hr at 56 ℃ and 90-110V. After electrophoresis, the gels were stained with SYBR-green I and II for 30 min. The DGGE band patterns were detected and documented with a gel documentation system (Oncor, Gaithersburg, MD).

Sequence analysis Amplicons were prepared by PCR such that a standard M13 primer site was incorporated at one end. These products were sequenced on an ABI 377 sequencer (Foster

City, CA) with kits containing Taq FS DNA polymerase and dye primer technology, as recommended by the manufacturer.

hMLH1 methylation analysis

DNA methylation patterns in the CpG islands of hMLH1 gene were determined by Methylation-specific PCR(MSP) as described[18]. The primer sequences of hMLH1 for unmethylated reaction were 5' -TTTTGATGTAGATGTTTTATTAGGGTTGT-3' (sense) and 5' -ACCACCTCAT CATAACTACCCACA-3' (antisense), and for methylated reaction were 5' -ACGTAGACGTTTTATT AGGGTCGC-3' (sense) and 5' -CCTCATCGTAACTACC CGCG-3' (antisense).

MSI detection

MSI analyses included five microsatellite markers: BAT25, BAT26, BAT40, D2S123, and D5S346. PCR was performed as previous described[1]. MSI was defined as the presence of band shift in the tumor DNA that was not present in the corresponding normal DNA. Based on the number of mutated MSI markers in each tumor, carcinomas were characterized as MSI-H if they manifested instability in two or more markers, MSI-L if unstable in only one marker, and MSS if they showed no instability in any marker[19,20].

Statistical analysis

Chi-square test with Yates' correction were used. A P value <0.05 was considered significant.

RESULTS

MSI in gastric cancer

Alterations of electrophoretic patterns of PCR products of five microsatellite markers were compared between the tumor and the normal DNA in each patient (Figure 1). MSI affecting at least one locus was observed in 17 (25 %) of 68 tumors, among which eight (11.8 %) were MSI-H, nine (13.2 %) were MSI-L, and fifty-one (75 %) were MSS.

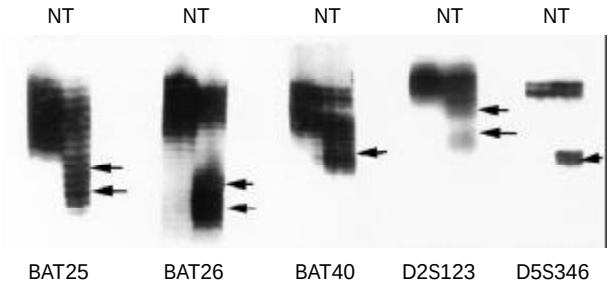

Figure 1 MSI in gastric cancer using 5 microsatellite loci (BAT-25, BAT-26, BAT-40, D2S123, and D5S346). Arrows indicate variant conformers. N: normal DNA pattern; T: tumor specimens containing variant conformers representing MSI.

hMLH1 mutation and MSI

Mutations and sequence alteration in various exons manifested as the four-spot pattern denoting a heterozygous variant (Figure 2). We found three mutations in 68 (4.4 %) gastric cancer by DNA sequencing. Two mutations were caused by a single bp substitution (exon 8 at codon 226, CGG→CTG, Arg→Leu; exon 9 at codon 252, TCA→TTA, Ser→Phe);. One identical change was caused by a 2 bp substitution (exon 12 at codon 409, CAG→CGT, Gln→Arg), which displayed similar DGGE band pattern. A comparison of MSI status with hMLH1 mutation was shown in Table 3. Three hMLH1 mutations were all detected in MSI-H, but no mutation was found in MSI-L or MSS.

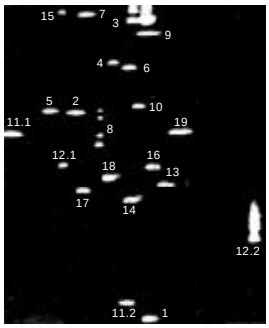

Figure 2 Detection of mismatching repair gene hMLH1 mutation in gastric cancer by two-dimentional DNA electrophoresis. Four-band pattern was observed at exon 8.

Table 3 The relevance of MSI and hMLH1 mutation

MSI Status	No.of cases	hMLH1 mutation
MSI-H	8	3
MSI-L	9	0
MSS	51	0

hMLH1 methylation status and MSI

To examine methylation of promoter region of hMLH1, we adapted MSP for the 5' CpG islands in this gene. The region chosen spaned the area of greatest CpG density immediately 5' to the transcription starting site, in an area previously studied for methylation changes[15]. In gastric mucosal samples without cancer, only unmethylated hMLH1 genes were present. Eleven of 68 (16.2 %) gastric cancers exhibited prominent methylation, which always had both methylated and nonmethylated hMLH1 genes (Figure 3). A comparison of MSI status with hMLH1 methylation status was shown in Table 4. Seven of 8 (87.5 %) cancers with MSI-H exhibited prominent methylation, whereas methylated hMLH1 gene was only found in 1/9 (11.1 %) gastric cancer with MSI-L and 3/51 (5.9 %) with MSS, suggesting that hMLH1 methylation was more correlated with gastric cancers with MSI -H than that with MSI -L or with MSS (P<0.01-0.001).

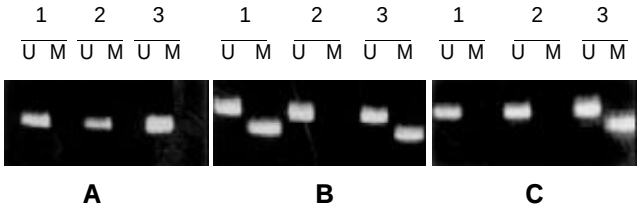

Figure 3 Methylation of hMLH1 in gastric cancer. A: No methylation was found in normal mucosa; B: Methylation of hMLH1 in MSI-H gastric cancer. Cases 1, 3 showed both hypermethylation and unmethylation; C: Methylation of hMLH1 in MSI-L gastric cancer. Case 3 showed both hypermethylation and unmethylation; U: Unmethylation; M: methylation.

Table 4 The relevance of methylation of hMLH1 and MSI

MSI status	Unmethylated	Hypermethylation(%)
MSI-H	1	7(87.5)
MSI-L	8	1(11.1)[b]
MSS	48	3(5.9)[d]

[b]P<0.01; [d]P<0.001 *vs* MSI-H group.

DISCUSSION

A significant portion of gastric cancers exhibit defective DNA mismatch repair, manifested as MSI. There is now evidence that MSI cancer comprises distinctive MSI-H and MSI-L categories[21-23]. MSI-H cancers are distingushed clinicopathologically and in their spectrum of genetic alterations from cancers showing MSI-L and MSS cancers[24-34]. Our previous studies indicated that MSI-H gastric cancers often showed lower frequency of LOH in APC, MCC and DCC genes than do MSI-L and MSS cancers[1]. In the present study, all three hMLH1 mutations were detected in MSI-H, but no mutation was found in those showing MSI-L or MSS. This result further indicates that hMLH1 is mutational target in MSI -H tumor cells and supported the notion that MIS-H tumors identified an alterative pathway of tumorigenesis that had been proposed by Vogelstein and co-workers[35].

Human cancers with MSI-H phenotype develop due to defects in DNA mismatch repair genes. Silencing of a DNA mismatch repair gene, hMLH1 gene, by promoter hypermethylation is a frequent cause of MSI-H phenotype[36-42]. In this study, 11 (16.2 %) of 68 gastric cancers exhibited prominent hMLH1 methylation, which is similar to previous studies[36]. It has been reported that MSI-H is related to methylation of the hMLH1 promoter but not hMSH1 mutations in sporadic gastric carcinomas[43]. It was also found that MSI-L gastric carcinomas share the hMLH1 methylation status of MSI-H carcinomas but not their clinicopathological profile[44]. In this study, 7/8 (87.5 %) cancers with MSI-H exhibited prominent methylation of the hMLH1 promoter, suggesting that methylation of the hMLH1 promoter is correlated with MSI-H gastric cancer. The frequency of methylation in MSI-H cancers is significantly higher than that in cancers with MSI-L and MSS, however no difference was found between cancers with MSI-L and MSS, indicating that MSI-H tumors identify a different methylation pathway from cancers with MSI-L and MSS, and MSI-L cancers involves the same methylation pathway as MSS tumors. This finding is in agreement with our recently published data on molecular pathway in the gastric carcinogenesis[2].

It has been found that colorectal cancer cell lines only had methylated hMLH1 genes, but had absent unmethylated hMLH1 genes[45]. In the present study, unlike the situation with the cell lines, however, the primary MSI+ gastric cancers always had both methylated and nonmethylated hMLH1 genes. It is likely that a significant fraction of the unmethylated genes is derived from the non-neoplastic cells (stromal, inflammatory, vascular, etc.), which are invariably present within the primary tumors but are not found in cultured cell lines. It has been reported that methylation of the hMLH1 promoter correlates with lack of expression of hMLH1 in sporadic colon tumors and mismatch repair-defective human tumor cell lines[13]. whereas demethylation of hMLH1 promoter results in reexpression of hMLH1 in tumor cells tested. Not only was the protein expressed, but MMR activity was restored. DNA methylation is a fundamental feature of the genomes and the control of their functions therefore it is a candidate for pharmacological manipulation that might have important therapeutic advantage.

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