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MSI-L Gastric Carcinomas Share the *hMLH1* Methylation Status of MSI-H Carcinomas but Not Their Clinicopathological Profile

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SUMMARY: Sporadic gastric carcinomas (SGC) with microsatellite instability (MSI) exhibit mutations in target genes and display a particular clinicopathological profile. In SGC the MSI phenotype has been associated with *hMLH1* promoter hypermethylation. Fifty-seven SGC, classified as high-frequency MSI (MSI-H), low-frequency MSI (MSI-L), and microsatellite stable (MSS), were analyzed for *hMLH1* promoter methylation status and clinicopathological features. *hMLH1* mutations and *hMLH1* expression, as well as target gene mutations, were also evaluated. Our aims were to characterize the molecular and clinicopathological features of SGC, with and without *hMLH1* promoter hypermethylation, and to compare the molecular and clinicopathological features of MSI-L, MSI-H, and MSS tumors in an attempt to clarify the place of MSI-L tumors in the mismatch repair (MMR) pathway. Hypermethylation of *hMLH1* promoter occurred in 27 of 57 SGC (47.3%) and was significantly associated with MSI status, target gene mutations, and expansive pattern of growth of the tumors. Seventy-five percent of the MSI-H and 50% of MSI-L carcinomas showed hypermethylation (Met+) of *hMLH1* in contrast to 0% in MSS carcinomas. No *hMLH1* expression was observed in MSI-L/Met+ and MSI-H/Met+ cases. MSS and MSI-L tumors share the same clinicopathological profile regardless of the methylation status of the latter and are distinct from MSI-H tumors. We conclude that mutations in target genes, more than hypermethylation or absence of expression of *hMLH1*, are the link between MSI status and most of the clinicopathological features of SGC. (*Lab Invest 2000, 80:1915–1923*).

Most tumors arising within the context of the hereditary nonpolyposis colorectal cancer (HNPCC) syndrome, as well as about 15% of sporadic colorectal carcinomas, exhibit a type of genetic instability characterized by the accumulation of ubiquitous somatic alterations in the length of simple repeated sequences (Ionov et al, 1993). This genome-wide instability of simple repeat sequences, referred to as microsatellite instability (MSI), is seen in 14% to 39% of sporadic gastric carcinomas (SGC) (Fleisher et al, 1999; Halling et al, 1999; Kang et al, 1999; Leung et al, 1999; Oliveira et al, 1998; Santos et al, 1996; Yamamoto et al, 1999).

The MSI phenotype, as found in HNPCC, is associated with defective DNA mismatch repair (MMR) genes, such as *hMLH1, hMSH2, hMSH3,* and *hMSH6*, among others (Liu et al, 1995; Wu et al, 1997, 1999). At variance with this, mutations in MMR genes are rare in sporadic colorectal and gastric carcinomas with the MSI phenotype (Borresen et al, 1995; Liu et al, 1995; Moslein et al, 1996; Wu et al, 1997; Yamamoto et al, 1999).

An alternative mechanism to mutations for silencing gene expression is hypermethylation of the gene promoter (Costello et al, 2000; Jones et al, 1999). *hMLH1* hypermethylation, with associated decreased protein expression, has been described in 44% to 100% of gastric carcinomas displaying a high level of MSI (MSI-H) (Fleisher et al, 1999; Leung et al, 1999; Suzuki et al, 1999; Toyota et al, 1999; Yamamoto et al, 1999). By contrast, this association has not been observed in microsatellite stable (MSS) tumors (Leung et al, 1999; Toyota et al, 1999; Yamamoto et al, 1999). The relationship between *hMLH1* hypermethylation and MSI phenotype is less clear with regard to tumors displaying a low level of MSI (MSI-L): Leung et al (1999), Kang et al (1999), Yamamoto et al (1999) and Toyota et al (1999) reported the absence of hypermethylation of *hMLH1,* as well as normal protein expression, in this type of tumor. Fleisher et al (1999), however, reported the occurrence of *hMLH1* hypermethylation in five out

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of six MSI-L tumors with associated diminished *hMLH1* expression.

In a previous study, we reported that MSI SGC exhibit mutations in a series of target genes (*TGFβRII*, *IGFIIR*, and *BAX*) and display a particular clinicopathological profile (Oliveira et al, 1998): MSI-H tumors were found to be significantly associated with distal localization, Lauren's intestinal and atypical histotypes, and Ming's expansive pattern of growth. In the present study, we analyzed *hMLH1* promoter hypermethylation in a series of 57 SGC, stratified into MSI-H ($n = 28$), MSI-L ($n = 12$), and MSS ($n = 17$). In a subset of cases, *hMLH1* mutations and *hMLH1* expression, as well as target gene mutations, were also evaluated. Our aims were twofold: (a) to characterize the molecular and clinicopathological features of SGC, with and without *hMLH1* promoter hypermethylation, and (b) to compare molecular, namely, *hMLH1* promoter methylation and target gene mutations, and clinicopathological features of MSI-L, MSI-H, and MSS tumors.

Results

MSI Status versus Clinicopathological Features

The 57 tumors were classified as MSS ($n = 17$), MSI-L ($n = 12$), and MSI-H ($n = 28$) using the criteria described in the "Materials and Methods" section. The comparison between MSS and MSI-H tumors showed a significant association between the MSI-H phenotype and Lauren's intestinal and atypical histotypes ($p = 0.05$), Ming's expansive pattern of growth ($p = 0.04$), lower pathological tumor, nodes, metastases (pTNM) stage of the tumor ($p = 0.05$), and the presence of mutations in target genes ($p = 0.0001$).

Table 1 summarizes the molecular and clinicopathological data and compares the MSI-L with MSS and MSI-H tumors. MSI-L tumors did not significantly differ from MSS tumors (Table1). By contrast, MSI-L tumors differ significantly from MSI-H tumors with regard to those same parameters that were found to

Table 1. Comparison of the Molecular and Clinicopathological Features of MSI-L and Those of MSS and MSI-H Sporadic Gastric Carcinomas

Molecular and clinicopathological features	No. of cases	MSS ($n = 17$)	*p* value MSS vs MSI-L	MSI-L ($n = 12$)	*p* value MSI-L vs MSI-H	MSI-H ($n = 28$)
Age	57	63.8 ± 6.9	0.10	56.3 ± 16.1	0.08	64.6 ± 11.7
Male/Female	57	9:8	0.55	5:7	0.63	14:14
Mutations in target genes	50[a]					
−		15	NP	8	0.0001	4
+		0		0		23
Location	56[b]					
Antrum		11	0.15	6	0.06	23
Body		2		5		3
Cardia		4		1		1
Lauren's classification	57					
Intestinal		10	0.84	7	0.02	17
Diffuse		3		3		0
Atypical		4		2		11
Ming's classification	56[a]					
Expanding		8	0.77	5	0.03	21
Infiltrative		9		7		6
Vascular invasion	56[a]					
Absent		9	0.13	3	0.34	11
Present		8		9		16
Lymph node metastases	56[a]					
Absent		5	0.49	5	0.56	14
Present		12		7		13
PTNM stage	57					
IA		4		3		1
IB		2		1		13
II		5	0.65	1	0.05	4
IIIA		5		6		7
IIIB		1		1		3

MSI-L, low-level microsatellite instability; MSS, microsatellite stable; MSI-H, high-level microsatellite instability; PTNM, pathological tumor, nodes, metastases; NP, statistical analysis not possible.

[a] The missing cases were not classifiable for technical reasons.

[b] One operated stomach.

be different in the comparison of MSS and MSI-H tumors (Table 1).

hMLH1 Methylation Analysis

The *hMLH1* promoter region of 21 of the 28 MSI-H cases (75%) could not be digested by *Hpa*II, indicating that all four *Hpa*II restriction sites were methylated. Six of the 12 (50%) MSI-L cases show the same methylation status. None of 17 MSS cases had methylation of the *hMLH1* promoter region (Fig. 1). The DNA isolated from all these cases were sensitive to the digestion by *Msp*I enzyme. The DNA from the normal mucosas of the stomach were all totally digested by *Hpa*II. The association between the MSI phenotype and methylation status of the promoter region was statistically significant ($p = 0.0001$). Results are summarized in Table 2.

hMLH1 Methylation Status versus Clinicopathological Features

Data on the relationship between the clinicopathological features of the 57 SGC and the methylation status of *hMLH1* are summarized in Table 2. A significant association was found between the methylation status and Ming's classification ($p = 0.01$): the majority of expanding tumors showed hypermethylation (62%), whereas only a minority of infiltrative tumors presented this phenotype (27%).

hMLH1 Mutations

Mutations in all exons of *hMLH1* were screened for 21 cases (17 methylation+ and 4 methylation−). No germline or somatic mutations of the *hMLH1* gene were found in one MSS case, and no mutations were found in the 2 MSI-L and in the 18 MSI-H cases. Although we did not find any causative mutation in this gene for any of the cases analyzed, we did find two variants in the PCR amplicons of exons 8 (10/21, 48%) and 15 (9/21, 43%). Both these variants are common polymorphisms and were previously described by Liu et al (1995).

hMLH1 Gene Expression

hMLH1 mRNA expression was detected in all three MSS cases. In the 2 MSI-L cases and in 6 of the 10 MSI-H cases, where material was available for RNA isolation, there was no expression of *hMLH1*. In four MSI-H cases there was residual expression of *hMLH1* when compared with *GAPDH* (Table 3 and Fig. 2).

Mutations in Target Genes (TGFβRII, IGFIIR, BAX, and TCF4)

Mutations in target genes were screened in 50 of the 57 cases. The presence of mutations in target genes (*TGFβRII*, *IGFIIR*, *BAX*, and *TCF4*) was significantly associated with *hMLH1* promoter methylation ($p = 0.0001$) because 20 out of 23 (87%) cases positive for mutations in target genes showed hypermethylation of this MMR gene (Table 2). Six cases had no mutations in any of the target genes but had *hMLH1* promoter methylation; five of these six tumors were MSI-L and the remaining case was a MSI-H carcinoma.

The association between mutations of target genes and the methylation status of *hMLH1* promoter region was statistically significant for *TGFβRII* ($p = 0.0001$), *BAX* ($p = 0.003$), and *IGFIIR* ($p = 0.03$) and not significant for *TCF4* ($p = 0.34$) (Fig. 3).

Methylation Status of MSI-L Carcinomas versus Molecular and Clinicopathological Features

The two MSI-L/Met+ cases analyzed by reverse transcription-polymerase chain reaction (RT-PCR) showed absence of expression of *hMLH1* (Table 3). No mutations in target genes were observed in MSI-L cases (5 Met+ and 3 Met−) regardless of the presence or absence of *hMLH1*. The comparison between MSI-L/Met+ and MSI-L/Met− cases, regarding the clinicopathological features, did not yield any significant differences (data not shown).

Discussion

Hypermethylation of the *hMLH1* promoter occurs with a very high frequency in SGC exhibiting MSI. We detected an aberrant *hMLH1* promoter methylation in

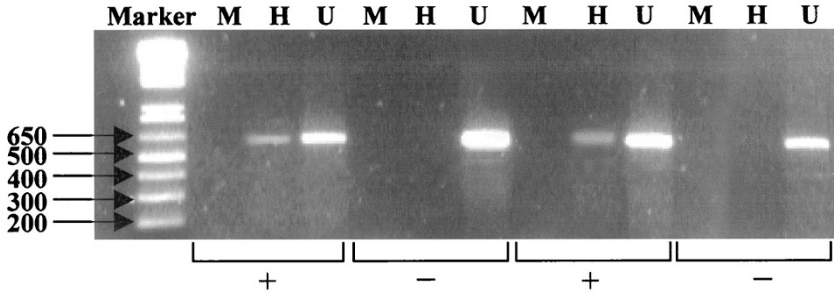

Figure 1.

Methylation of *hMLH1* promoter in gastric carcinomas. (+) Cases with hypermethylation of *hMLH1* promoter. The presence of PCR product in *Hpa*II digestion indicates a hypermethylated promoter region of *hMLH1*. (−) Cases without *hMLH1* promoter hypermethylation. The absence of PCR product in *Hpa*II digestion indicates a nonmethylated promoter region of *hMLH1*. The absence of PCR product in *MSPI* digestion indicates a complete digestion of DNA and serves as control. U, undigested; H, *Hpa*II-digested; M, *MSPI*-digested.

Table 2. Summary of the Molecular and Clinicopathological Features of 57 Sporadic Gastric Carcinomas Regarding the Methylation Status of the *hMLH1* Gene Promoter

Molecular and clinicopathological features	No. of cases	Met− (n = 30)	Met+ (n = 27)	p value
Age (mean ± SD)	57	61.2 ± 9.3	64.1 ± 14.2	0.35
Male/Female	57	17:13	11:16	0.23
MSI status	57			
MSS		17	0	
MSI-L		6	6	0.0001
MSI-H		7	21	
MLH1 expression	15			
−/↓		0	12	0.0001
+		3	0	
Mutations in target genes	50[a]			
−		21	6	0.0001
+		3	20	
Location	56[b]			
Antrum		19	21	
Body		7	3	0.35
Cardia		4	2	
Lauren's classification	57			
Intestinal		18	16	
Diffuse		5	1	0.21
Atypical		7	10	
Ming's classification	56[a]			
Expanding		13	21	0.01
Infiltrative		16	6	
Vascular invasion	56[a]			
Absent		14	9	0.26
Present		15	18	
Lymph node metastases	56[a]			
Absent		11	13	0.44
Present		18	14	
PTNM stage	57			
IA		6	2	
IB		5	11	
II		6	4	0.30
IIIA		10	8	
IIIB		3	2	

[a] The missing cases were not classifiable for technical reasons.
[b] One operated stomach.

75% of MSI-H gastric carcinoma cases leading to the absence or diminished expression of *hMLH1*. This percentage fits with those previously reported for SGC (Fleisher et al, 1999; Kang et al, 1999; Leung et al, 1999; Toyota et al, 1999; Wu et al, 2000; Yamamoto et al, 1999), sporadic colorectal carcinomas (Cunningham et al, 1998; Ghimenti et al, 1999; Kang et al, 1999), and endometrial carcinomas displaying MSI (Esteller et al, 1998; Simpkins et al, 1999). The RT-PCR analysis of *hMLH1* expression showed that there is a significant association between *hMLH1* hypermethylation and loss of *hMLH1* expression as previously reported by Deng et al (1999). Using a polymorphism described in the promoter region of *hMLH1*, we were able to determine that in three MSI-H cases the hypermethylation occurs as a biallelic event as previously found by Veigl et al (1998) (data not shown). The high degree of instability associated with microsatellite markers in MSI tumors prevented us from accurately assessing the loss of heterozygosity (LOH) status of the *hMLH1* gene in the tumors. Our results, together with data from literature, support the hypoth-

Table 3. Summary of the Data Regarding the Methylation Status of the Promoter, cDNA Expression, Mutations of *hMLH1* and the MSI Status in 15 of the 57 Sporadic Gastric Carcinomas

Case No.	Methylation status	Expression (RT-PCR *hMLH1*)	MLH1 mutations	MSI status
1	−	+	−	MSS
2	−	+	nd	MSS
3	−	+	nd	MSS
4	+	−	−	MSI-L
5	+	−	−	MSI-L
6	+	−	−	MSI-H
7	+	−	−	MSI-H
8	+	−	−	MSI-H
9	+	−	−	MSI-H
10	+	−	−	MSI-H
11	+	−	−	MSI-H
12	+	↓	−	MSI-H
13	+	↓	−	MSI-H
14	+	↓	nd	MSI-H
15	+	↓	−	MSI-H

− (minus), absence of expression; + (plus), expression of *hMLH1*; ↓, residual expression; nd, not determined.

esis that *hMLH1* promoter hypermethylation is the most prevalent mechanism of MMR deficiency in SGC.

In 7 of the 28 MSI-H cases, no *hMLH1* hypermethylation was observed, suggesting an alternative mechanism for *hMLH1* inactivation in these cases. Somatic mutations in one of the MMR genes have been detected in up to 26% of sporadic MSI cancers at various sites, namely colon and endometrium (Borresen et al, 1995; Bubb et al, 1996; Katabuchi et al, 1995; Kowalski et al, 1997; Liu et al, 1995; Moslein et al, 1996; Thibodeau et al, 1996; Wu et al, 1997). Data on MMR gene mutations is scarce in SGC. Yamamoto et al (1999) showed the presence of *hMSH2* and *hMLH1* somatic mutations in, respectively, 6 and 1 out of 24 cases of MSI-H SGC. In our series we did not find *hMLH1* mutations in any of the tumors, including the four MSI-H cases without hypermethylation that we were able to screen. Therefore, the mechanism of *hMLH1* inactivation in hypermethylation/mutation-negative gastric carcinomas remains to be elucidated.

We observed a significant association between *hMLH1* promoter methylation and mutations in target genes. We think that this finding may be ascribed, partly at least, to the close relationship between MSI-H and methylation status.

We found a significant association between *hMLH1* hypermethylation and Ming's classification: *hMLH1* hypermethylation was detected in 62% of the expanding tumors and in 27% of the infiltrative tumors. If this finding is confirmed in a larger series, one might have to consider the possibility that target genes other than those we looked at are linked to the expanding growth pattern of the tumors. It is tempting to advance that the "new" target genes may be involved in cell-cell or cell-matrix adhesion. No other significant associations were found between *hMLH1* hypermethylation and clinicopathological features. The discrepancy between the clinicopathological features of the MSI-H cases and those of cases with *hMLH1* hypermethylation may be explained by two facts: the group of tumors with *hMLH1* hypermethylation includes some MSI-L tumors and not all MSI-H tumors present *hMLH1* hypermethylation.

In MSI-L gastric carcinomas, we found *hMLH1* hypermethylation in 50% of the tumors. These results are in agreement with those reported by Fleisher et al (1999) in gastric carcinoma and by Esteller et al (1999) in endometrial carcinoma. There are no significant differences between MSS and MSI-L tumors as regards their clinicopathological profile. Toyota et al (1999) suggested that tumor development in cases presenting the MSI-L phenotype may be accompanied by random de novo hypermethylation. This hypermethylation, however, confers no selective advantage to the tumor cells. Curiously, if this hypothesis is true, MSS tumors should also have presented a proportion of cases with aberrant methylation of the *hMLH1* promoter, but we did not observe this in our series. Furthermore, in two MSI-L cases in which *hMLH1* expression was evaluated, we observed that *hMLH1* promoter hypermethylation led to a loss of *hMLH1* expression. The aforementioned facts suggest that *hMLH1* hypermethylation does not occur merely by chance in MSI-L gastric carcinomas.

The possibility that MSI-L/Met+ cases represent an intermediate step in the MMR pathway has been advanced by Esteller et al (1999) regarding the occurrence of early endometrial carcinomas. This possibility does not fit with the results obtained in the present series: only two of the six MSI-L/Met+ cases were tumors restricted to the submucosa (early carcinomas), whereas the remaining four cases were advanced tumors. Because it is difficult to conceive that these MSI-L/Met+ advanced gastric carcinomas might be seen as cases not yet expressing a complete mutator phenotype, we would rather suggest that these tumors may have followed (an) unknown MMR-independent molecular pathway(s) of progression.

In biological terms we think that the distinction between MSI-L/Met+ gastric cancers and MSS tumors should be made because the aforementioned subset of MSI-L tumors share the same genetic alterations of MSI-H tumors: hypermethylation of the promoter region and absence of expression of *hMLH1*. For practical purposes, no benefit appears to be achieved by making a distinction between MSS and MSI-L tumors because they share the same clinicopathological profile. Our results show that mutations in target genes, more than hypermethylation or absence of expression of *hMLH1*, are the link between MSI status and most of the clinicopathological features of SGC.

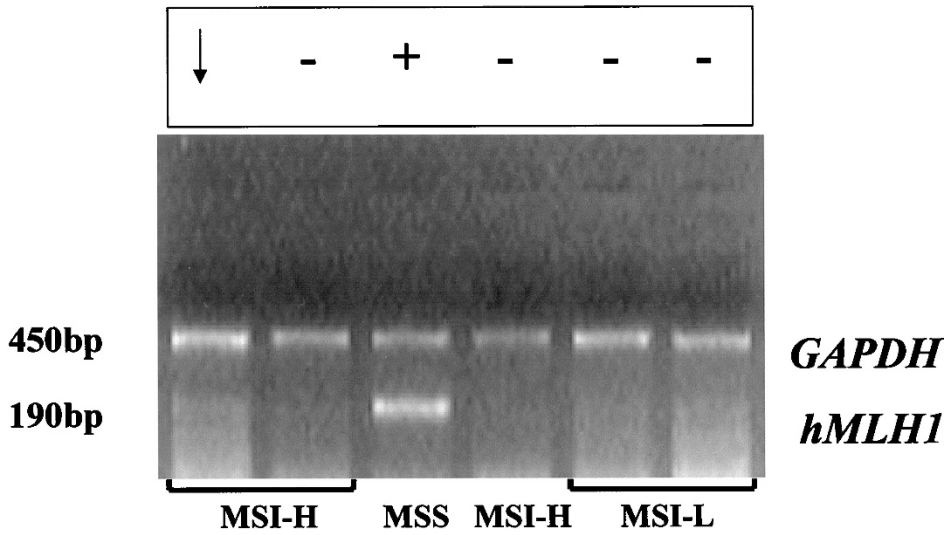

Figure 2.
Expression of *hMLH1* MSS, MSI-L, and MSI-H gastric carcinomas. (−) Cases with absence of expression of *hMLH1*. (+) Cases with *hMLH1* expression. (↓) Cases with residual expression of *hMLH1* in comparison with *GADPH*.

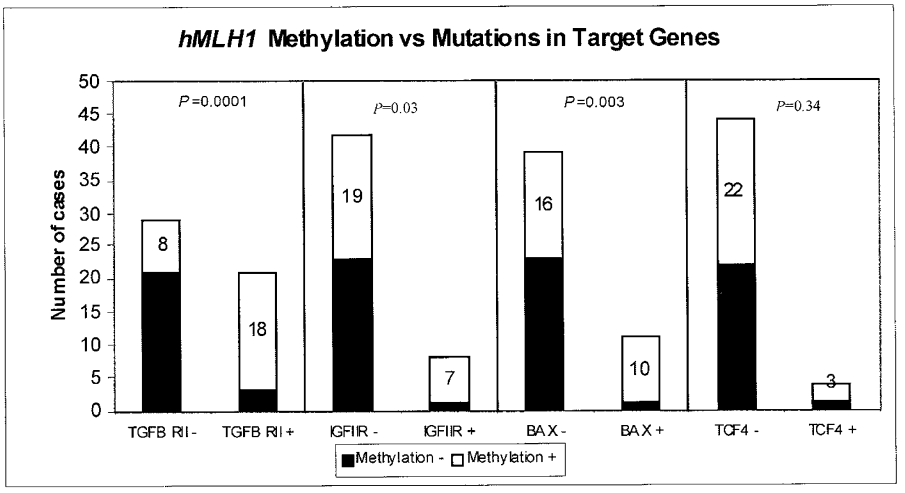

Figure 3.
Relationship between methylation of the promoter region of *hMLH1* gene and the occurrence of mutations in the repetitive sequences of target genes: *TGFβRII*, *IGFIIR*, *BAX*, and *TCF4*.

Materials and Methods

Patients, Tissue Samples, and DNA Extraction

In this study we analyzed 57 tumors selected from a series of 152 gastric carcinomas consecutively resected at Hospital of S. João (Porto, Portugal) from 1988 to 1997, previously analyzed for MSI phenotype and, partly, for mutations in target genes (Oliveira et al, 1998). We analyzed all cases with MSI-H or MSI-L phenotype (see below) after excluding 16 cases because of the lack of good high-quality DNA and 10 cases because of insufficient material. We also analyzed a control group of 17 cases with MSS phenotype selected at random from the cases of MSS with good technical conditions. Hematoxylin and eosin-stained sections were used to classify the tumors according to Lauren's classification. The pathological staging was achieved using the unified 1987 tumor, node, metastasis (TNM) system for gastric carcinoma. Orcein-stained sections were used for the detection of vascular invasion.

MSI Assays

The 57 gastric carcinomas were previously studied for MSI using a panel of at least five dinucleotide repeat sequences, as described by Santos et al (1996), and using a primer set localized on intron 5 of the *hMSH2* gene, which amplifies an adenine quasi monomorphic mononucleotide repeat, BAT26, as described by Zhou et al (1998) and Oliveira et al (1998).

Cases were classified as having an MSI-H phenotype whenever they presented a high frequency of MSI

(≥ 40%) and BAT26+. Cases were considered as MSI-L whenever they showed instability in less than 40% of the markers used. MSS cases showed no instability at any of the markers used.

Promoter Methylation Assays

The promoter methylation is analyzed as initially described by Kane et al (1997). In short, DNA is digested by *Hpa*II or *Msp*I, methylation sensitive and insensitive enzymes, respectively, after which PCR is performed amplifying the target region. Absence or presence of PCR product indicates nonmethylated or methylated promoter region, respectively. Tumor DNA samples were digested over 48 hours at 37° C in 100 μl total volume reactions, containing 250 ng of genomic DNA of each sample, no enzyme, 100 units of *Hpa*II or 150 units of *Msp*I. Five normal mucosas (distal to the tumor) of the stomach were analyzed as controls. To analyze the cleavage of the *hMLH1* promoter region, 1 μl of each digest was amplified by PCR in 30 μl reactions containing 3 μl of 10× PCR reaction buffer (Pharmacia Biotech, Piscataway, New Jersey), 0.5 units of *Taq* polymerase enzyme (Pharmacia Biotech), 0.25 μM of each of the four deoxynucleotide triphosphates, and 0.4 μM of each primer (5′-CGCTCGTAGTATTCGTGC-3′ and 5′- TCAGTGCCTCGTGCTCAC-3′), designed to amplify nucleotides −670 to −65 of *hMLH1* (Genbank Accession No. U83845). Thirty-five cycles of PCR were performed using the following conditions: 94° C, 30 seconds; 55° C, 30 seconds; 72° C, 120 seconds. The resulting amplification products of two independent experiments were analyzed by 2% agarose gel electrophoresis using standard conditions.

Mutation Analysis of hMLH1

Mutational analysis of *hMLH1* gene was performed by denaturing gradient gel electrophoresis (DGGE). Multiplex PCR was carried out on 500 ng of DNA in a total volume of 30 μl for 35 cycles as follows: denaturing step at 94° C for 3 minutes; 5 cycles consisting of denaturation at 94° C for 1 minute, annealing at 56° C for 1 minute, and elongation at 72° C for 1 minute; 5 cycles consisting of denaturation at 94° C for 1 minute, annealing at 53° C for 1 minute, and elongation at 72° C for 1 minute; and 25 cycles each of denaturation at 94° C for 1 minute, annealing at 50° C for 1 minute, and elongation at 72° C for 1 minute; one final elongation cycle was performed at 72° C for 5 minutes. The PCR mixture contained 1× *Taq* reaction buffer, 1 mM $MgCl^2$, 0.5 unit of *Taq* polymerase, 0.75 mM of deoxyribonucleoside triphosphate (dNTP), and 15 pmol of each primer. The primers used for amplification of *hMLH1* gene are listed in the literature (Wu et al, 1997), along with fragment sizes, melting temperatures, and PCR-annealing temperatures. To increase the amount of heteroduplex molecules, a heteroduplex step was performed after PCR amplification, ie, the samples were denatured for 10 minutes at 96° C followed by renaturation for 45 minutes at 50° C. PCR products were loaded on a polyacrylamide gel containing a denaturing gradient of urea/formamide (UF) (10% UF to 70% UF). Electrophoresis was performed overnight in TAE buffer at 59° C. Gels were stained with ethidium bromide and photographed under a UV transilluminator.

Expression Analysis of hMLH1

RT-PCR studies were performed using frozen material from 15 cases (10 MSI-H, 2 MSI-L, 3 MSS). Total RNA was extracted using standard methods. First-strand synthesis was made by random 6-mer priming using M-MLV reverse transcriptase (Boehringer Mannheim, Lewes, United Kingdom) at 37° for 60 minutes. *hMLH1* mRNA expression level analysis was performed by co-amplification of the target gene (*hMLH1*) and of the housekeeping gene *GAPDH*.

Amplification of the Target Genes (TGFβRII, IGFIIR, BAX, and TCF4)

Detection of mutations in target genes was performed in 50 cases (27 MSI-H, 8 MSI-L, and 15 MSS) in which constitutional and tumor DNA was available. Of the 50 cases analyzed for *TGFβRII*, *IGFIIR*, and *BAX*, 34 had been analyzed previously by Oliveira et al (1998). The repeat sequence $(A)_9$ in the putative exon 10 of *TCF4* could only be analyzed in 48 cases as described by Duval et al (1999). PCR products were analyzed for mutations in 6% single mutation detection enhancement/strand conformation polymorphism analysis (MDE/SSCP) gels. The results were confirmed with radioactive PCR, and the products were run in a 10% denaturing PAA sequencing gel. The tumors were classified as harboring a mutation whenever they showed band shifts relative to the normal tissue.

Statistical Analysis

The statistical analysis of the results was performed using the χ^2 test with Yates correction, Fisher's exact test, and Student's *t* test. The parameters used for this analysis are listed in Tables 1 and 2. A *p* value less than 0.05 was considered statistically significant.

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