



## Hypermethylation of the *hMLH1* gene promoter is associated with microsatellite instability in early human gastric neoplasia

A Steven Fleisher<sup>1,8</sup>, Manel Esteller<sup>5,8</sup>, Gen Tamura<sup>6</sup>, Asma Rashid<sup>3</sup>, O Colin Stine<sup>3</sup>, Jing Yin<sup>1</sup>, Tong-Tong Zou<sup>1</sup>, John M Abraham<sup>1</sup>, Dehe Kong<sup>1,4</sup>, Satoshi Nishizuka<sup>7</sup>, Stephen P James<sup>1</sup>, Keith T Wilson<sup>1,2</sup>, James G Herman<sup>5</sup> and Stephen J Meltzer<sup>\*1,2,4</sup>

<sup>1</sup>Department of Medicine, Gastroenterology Division, University of Maryland School of Medicine and Baltimore VA Hospital, Baltimore, Maryland, MD 21201, USA; <sup>2</sup>Greenebaum Cancer Center, University of Maryland School of Medicine and Baltimore VA Hospital, Baltimore, Maryland, MD 21201, USA; <sup>3</sup>Program in Genetics, University of Maryland School of Medicine and Baltimore VA Hospital, Baltimore, Maryland, MD 21201, USA; <sup>4</sup>Department of Pathology, University of Maryland School of Medicine and Baltimore VA Hospital, Baltimore, Maryland, MD 21201, USA; <sup>5</sup>The Johns Hopkins Oncology Center, Baltimore, Maryland, MD 21231, USA; <sup>6</sup>Department of Pathology, Yamagata University School of Medicine, Yamagata 990, Japan; <sup>7</sup>Department of Microbiology and Molecular Genetics, University of California, Irvine, California, USA

A significant portion of gastric cancers exhibit defective DNA mismatch repair, manifested as microsatellite instability (MSI). High-frequency MSI (MSI-H) is associated with hypermethylation of the *human mut-L homologue 1 (hMLH1)* mismatch repair gene promoter and diminished *hMLH1* expression in advanced gastric cancers. However, the relationship between MSI and *hMLH1* hypermethylation has not been studied in early gastric neoplasms. We therefore investigated *hMLH1* hypermethylation, *hMLH1* expression and MSI in a group of early gastric cancers and gastric adenomas. Sixty-four early gastric neoplasms were evaluated, comprising 28 adenomas, 18 mucosal carcinomas, and 18 carcinomas with superficial submucosal invasion but clear margins. MSI was evaluated using multiplex fluorescent PCR to amplify loci D2S123, D5S346, D17S250, BAT 25 and BAT 26. Methylation-specific PCR was performed to determine the methylation status of *hMLH1*. In two hypermethylated MSI-H cancers, *hMLH1* protein expression was also evaluated by immunohistochemistry. Six of sixty-four early gastric lesions were MSI-H, comprising 1 adenoma, 4 mucosal carcinomas, and 1 carcinoma with superficial submucosal invasion. Two lesions (one adenoma and one mucosal carcinoma) demonstrated low-frequency MSI (MSI-L). The remaining 56 neoplasms were MSI-stable (MSI-S). Six of six MSI-H, one of two MSI-L, and none of thirty MSI-S lesions showed *hMLH1* hypermethylation ( $P < 0.001$ ). Diminished *hMLH1* protein expression was demonstrated by immunohistochemistry in two of two MSI-H hypermethylated lesions. *hMLH1* promoter hypermethylation is significantly associated with MSI and diminished *hMLH1* expression in early gastric neoplasms. MSI and hypermethylation-associated inactivation of *hMLH1* are more prevalent in early gastric

cancers than in gastric adenomas. Thus, hypermethylation-associated inactivation of the *hMLH1* gene can occur early in gastric carcinogenesis. *Oncogene* (2001) 20, 329–335.

**Keywords:** hypermethylation; *hMLH1*; microsatellite instability; mismatch repair; gastric cancer; gastric neoplasia

### Introduction

Gastric cancer is the second most common cancer worldwide: estimated crude incidence rates indicate that it accounts for 9.9% of all cancers (Parkin *et al.*, 1999; Savides, 1999). Its etiology is unknown, but strong associations exist with several dietary or environmental factors and with gastric mucosal infection by the bacterium *Helicobacter pylori* (Craanen *et al.*, 1992; Parsonnet *et al.*, 1991).

Microsatellite instability (MSI) comprises length mutations in tandem oligonucleotide repeats which occur in a large subset of human tumors (Han *et al.*, 1993; Orlow *et al.*, 1994; Rhyu *et al.*, 1994; Thibodeau *et al.*, 1993). This type of mutation is believed to be caused by deficient DNA mismatch repair (MMR) (Thibodeau *et al.*, 1996; Aaltonen *et al.*, 1993; Ionov *et al.*, 1993; Leach *et al.*, 1996). Among human sporadic tumors, gastric carcinoma possesses the highest prevalence of MSI, with up to 44% of cases manifesting this abnormality (Rhyu *et al.*, 1994; Akiyama *et al.*, 1996; Keller *et al.*, 1998; Ottini *et al.*, 1997). The underlying cause of MSI in gastric tumors is unknown: primary mutations in major MMR genes, e.g., *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 (Yin *et al.*, 1997). 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 (Esteller *et al.*,

\*Correspondence: SJ Meltzer, University of Maryland, 22 S. Greene St, Room N3W62, Baltimore, MD 21201, USA

<sup>8</sup>The first two authors contributed equally to this work

Received 29 August 2000; revised 8 November 2000; accepted 9 November 2000

1998; Herman *et al.*, 1998; Cunningham *et al.*, 1998; Kane *et al.*, 1997; Esteller *et al.*, 1999a). Epigenetic methylation-associated inactivation of genes is not limited to the *hMLH1* MMR gene. Hypermethylation of normally unmethylated CpG islands in the promoter regions of multiple tumor suppressor genes and DNA repair genes, including p16<sup>INK4a</sup>, p14<sup>ARF</sup>, p15<sup>INK4b</sup>, VHL, E-cadherin, GSTP1 and the DNA repair gene MGMT suggest an alternative mechanism for tumor suppressor gene inactivation (Baylin and Herman, 2000; Esteller *et al.*, 2000; Esteller *et al.*, 1999b). We and others have demonstrated hypermethylation-associated inactivation of *hMLH1* in high-frequency MSI (MSI-H) gastric cancers at advanced clinical stages (Fleisher *et al.*, 1999; Leung *et al.*, 1999; Kang *et al.*, 1999; Suzuki *et al.*, 1999). In addition, we found that 6/8 low-frequency MSI (MSI-L) gastric cancers at advanced clinical stages were hypermethylated at *hMLH1*.

Very few studies have addressed the relationship of MSI to *hMLH1* promoter methylation in early neoplasms. In uterine endometrial hyperplasia, a precursor lesion to uterine cancer, a relationship between MSI and *hMLH1* hypermethylation has been demonstrated (Esteller *et al.*, 1999a). No previous reports have addressed *hMLH1* hypermethylation in early gastric lesions. Gastric adenomas sometimes coexist with gastric adenocarcinomas, and the risk of carcinoma may be increased in patients with gastric adenomas (Tomasulo *et al.*, 1971). The reported frequency of MSI in gastric adenomas ranges from 14–30% (Isogaki *et al.*, 1999; Semba *et al.*, 1996). In one report, six of seven adenomas demonstrating instability were MSI-L (Isogaki *et al.*, 1999). However, the progression of gastric adenomas through the adenoma-carcinoma sequence has been disputed (Semba *et al.*, 1996; Tamura, 1996). We studied adenomas for MSI and *hMLH1* methylation, not only for their molecular characterization, but also in order to help resolve this controversy. We hypothesized that the frequencies of MSI and *hMLH1* hypermethylation in adenomas should resemble those found in early gastric neoplasms. We further hypothesized that MSI-L is an 'intermediate' form of MSI which occurs earlier during carcinogenesis than MSI-H; thus, MSI-L should predominate in early gastric neoplasms.

Therefore, we sought to determine: (1) how frequently hypermethylation of *hMLH1* occurs in early gastric neoplasms (i.e., adenomas and carcinomas), and how strongly it is associated with MSI; (2) whether MSI-L is more frequent than MSI-H in early gastric neoplasms.

## Results

### *Microsatellite instability*

MSI status had already been tested on 24 of 28 adenomas and in one early gastric cancer. Of the 24 adenomas, compared with matched normal control tissue, one (S10) was floridly MSI-H (with 20 of 35

loci showing mutations), while another (RCH-5) was barely MSI-L (with only one of 35 loci mutated) (Tamura, 1996). The one previously tested early gastric cancer was MSI-H, with mutations in 12/28 loci (Fleisher *et al.*, 1999). The remaining four adenomas and 35 early gastric cancers in our study were tested for MSI as described above. Here, four of four adenomas were MSI-S, whereas the early cancers showed MSI-H in four of 35 (11.4%), MSI-L in one of 35 (2.9%) and MSI-S in 30 of 35 (86.7%). When taken together, two of 28 adenomas (7.1%) and six of 36 early cancers (16.7%) showed either low or high MSI (Table 1). Results of these MSI studies are summarized in Table 1, while examples of MSI determined by fluorescent labeled sequencing are depicted in Figure 1.

### *Methylation-specific PCR*

Results of *hMLH1* promoter hypermethylation assays *versus* MSI studies in early gastric neoplasms are summarized in Table 1 and illustrated in Figure 2. *hMLH1* promoter methylation was determined in all samples except the 24 MSI-S adenomas. Hypermethylation of the *hMLH1* promoter occurred in six of six MSI-H cases (100%) and one of two MSI-L cases (50%). Taking these groups together, seven of eight cases (87.5%) showing either low or high MSI were hypermethylated. In contrast, none of thirty cases that were MSI-S exhibited *hMLH1* promoter hypermethylation ( $P < 0.0001$  for MSI-H plus MSI-L *versus* MSI-stable, Fisher's exact test, two-tailed). When MSI-H cases were compared with MSI-L and MSI-S together or with MSI-S alone, both comparisons significantly associated *hMLH1* hypermethylation with high MSI ( $P < 0.0001$ ). If the gastric adenomas were excluded from the analysis, the association of MSI-H early gastric cancers with *hMLH1* hypermethylation remained highly significant ( $P < 0.0001$  for MSI-H early gastric cancers *versus* MSI-S early gastric cancers alone or *versus* MSI-L plus MSI-S early gastric cancers). In addition, seven (100%) of an additional group of seven MSI-S adenomas assayed showed no hypermethylation of the *hMLH1* promoter.

### *Immunohistochemistry*

Two cases (M112 and M281) showing *hMLH1* promoter hypermethylation and MSI-H were tested for *hMLH1* nuclear protein expression using immunohistochemistry. These cases showed diminished *hMLH1* expression in tumor cell nuclei relative to normal cell nuclei in the same section. Results of staining are displayed in Figure 3.

## Discussion

The above data show that *hMLH1* promoter hypermethylation is significantly associated with widespread MSI (MSI-H) in early gastric neoplasms. In this

**Table 1** Microsatellite instability and *hMLH1* hypermethylation in early gastric neoplasia

Pathology	Sample numbers	MSI status <sup>a</sup>	<i>hMLH1</i> methylation status
Adenomas	S10	MSI-H	Hypermethylated
	RCH-5	MSI-L	Unmethylated
	B1-3, B5-9, PB1, PB3, PB5, S2, S4-5, S8-9, S11-12, RCH-1, RCH-3, P3-4, OGA	MSI-S	
Mucosal Cancers	M112, M256, M245 and M132	MSI-H	Hypermethylated
	M207, M221, M228, M243, M252, M264, M34 and M153	MSI-S	Unmethylated
Diffuse	M274	MSI-L	Hypermethylated
	M188, M247, M267, M169 and M176	MSI-S	Unmethylated
Submucosal Cancers <sup>b</sup>	M281	MSI-H	Hypermethylated
	M33, M218, M222, M225, M241, M249, M253, M257, M260, M269, M280	MSI-S	Unmethylated
Diffuse	M165, M258, M261, M268, M158, M146	MSI-S	Unmethylated

<sup>a</sup>Tumors were characterized as MSI-H if they manifested at two or more loci (or greater than 30% of loci if more than five loci were tested), MSI-L if unstable at only one locus (or less than 30% of loci if more than five loci were tested), and MSI-S if showing no instability at any loci.

<sup>b</sup>Carcinomas with superficial submucosal invasion but clear margins

regard, early gastric tumors resemble advanced MSI-H gastric cancers (Fleisher *et al.*, 1999; Leung *et al.*, 1999; Kang *et al.*, 1999; Suzuki *et al.*, 1999). The concurrence of *hMLH1* hypermethylation with diminished *hMLH1* protein expression in the current study suggests an association of *hMLH1* hypermethylation with transcriptional inactivation and consequent MMR deficiency in early gastric neoplasia. Indeed, current literature suggests that *hMLH1* hypermethylation is the predominant mechanism underlying MMR deficiency in sporadic human tumors. In support of this hypothesis, hypermethylation of other MMR genes such as *hMSH2*, *hMSH3*, and *hMSH6* in endometrial cancer and *hMSH2* in colorectal cancer has not been demonstrated (Esteller *et al.*, 1998, 1999a). In addition, the present early gastric neoplasia data are consistent with previous reports of *hMLH1* hypermethylation in advanced colorectal, endometrial, and gastric cancers manifesting MSI (Esteller *et al.*, 1998; Herman *et al.*, 1998; Cunningham *et al.*, 1998; Fleisher *et al.*, 1999; Iino *et al.*, 1999).

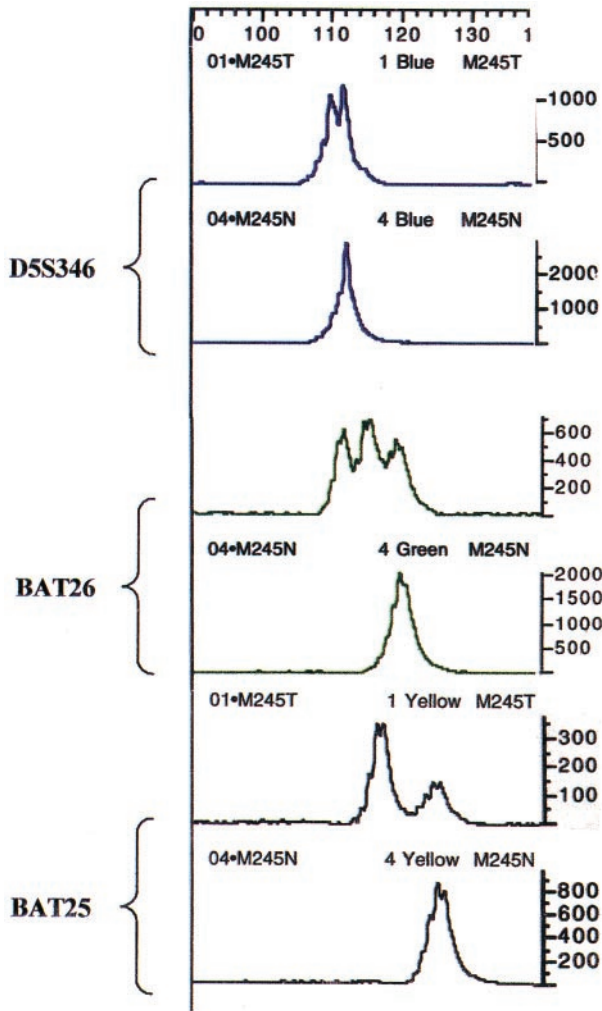
The overall frequency of MSI in this study was slightly lower than that in some published series, perhaps because of its greater preponderance of early lesions. Moreover, there was no association between MSI and stage or grade. For example, 1/8 adenomas with mild atypia, 1/6 with moderate atypia, and 0/1 with severe atypia showed MSI ( $P = 1.00$ , Fisher's exact test, two-tailed).

The optimal approach to determining mismatch repair deficiency phenotype has been the subject of some debate. An NIH panel recommended the use of five instability loci which include both mono- and dinucleotide repeat sequences (Boland *et al.*, 1998). On the other hand, others suggested a need for testing fewer mononucleotide markers (Zhou *et al.*, 1998; Hoang *et al.*, 1997). In gastric cancer, the possibility of testing for MSI using the mononucleotide repeat BAT 26 alone has been raised (Halling *et al.*, 1999). Rapid, inexpensive MSI testing could offer more than just

molecular insights: it could serve as a biomarker in treatment algorithms. For example, *in vitro* studies suggest that MMR-deficient cancers are more tolerant to the DNA damage caused by methylating chemotherapeutic agents, and thus less likely to respond to these agents (Karran and Hampson, 1996; Claij and te Riele, 1999). In this context, it is interesting to note that all of our early cancers with MSI (-H and -L), as well as the single MSI-H adenoma, showed instability at the dinucleotide repeat locus D2S123. Furthermore, all lesions with instability at D2S123 were hypermethylated at *hMLH1*. We repeat-tested MSI status using fluorescent primers and automated gel analysis in 46 advanced gastric cancers (data not shown). Of these advanced gastric cancers, 11/13 MSI-H and 2/6 MSI-L lesions showed MSI at the dinucleotide locus D2S123. In contrast, 8/13 MSI-H and 1/6 MSI-L lesions showed MSI at the mononucleotide repeat BAT 26. Although these numbers are somewhat small, they suggest that D2S123 may be more sensitive than BAT 26 as an MSI screening locus in gastric cancers. If reproduced in larger series, this finding could establish D2S123 as an effective MMR deficiency marker in gastric cancer patients.

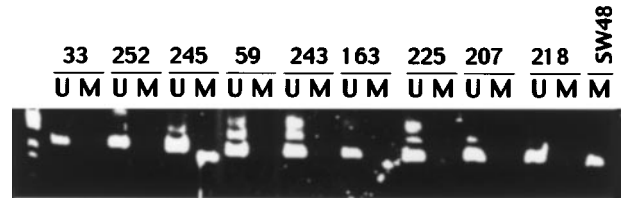
Another interesting point concerns our finding that 4/5 MSI-H early gastric cancers (80%) were histologically the intestinal type, rather than the diffuse or scattered variety. Intestinal histopathology has been associated with MSI (Ottini *et al.*, 1997). Studies have also shown a correlation between *H. pylori* infection and intestinal-type gastric cancer (Driessen *et al.*, 1999; Kuipers, 1998; Wu *et al.*, 1997). Thus, it is possible that *H. pylori* predisposes to *hMLH1* hypermethylation and MMR deficiency.

Published frequencies of MSI-H in gastric cancer range between 13–44% (Rhyu *et al.*, 1994; Keller *et al.*, 1998; Ottini *et al.*, 1997; dos Santos *et al.*, 1996; Halling *et al.*, 1999). Thus, our finding of MSI-H in 5/36 early gastric cancers (13.8%) is in keeping with published frequencies. However, only 1/28 adenomas



**Figure 1** Representative result of microsatellite instability analysis using fluorescently labeled primers after electrophoresis on an ABI Prizm automated sequencer, with generation of plot by Genotyper software. Depicted are three microsatellite loci (D5S346, BAT 26, and BAT 25) showing MSI in a patient (M245) with widespread instability (MSI-H). Number of bases is displayed on the horizontal axis at the top of the plot. The vertical axes represent arbitrary fluorescent units. The color of the fluorescent marker is annotated and displayed. The yellow fluorescent tracing is displayed in black for ease of visualization. Note the additional, abnormal peak(s) in tumor DNA compared with matched normal control DNA

(3.57%) were MSI-H. Assuming that *hMLH1* methylation is an early oncogenic event, we would have expected gastric cancer precursor lesions to manifest MSI at frequencies similar to those in full-blown cancers. We therefore propose that gastric adenomas are *not* precursor lesions. This contention is supported by epidemiological studies suggesting that the intestinal type of gastric cancer originates from intestinal metaplasia, rather than from adenomas (Correa and Shiao, 1994). Additional support is lent to this hypothesis by the finding of intestinal metaplastic tissue adjacent to intestinal type gastric cancers (Sasaki et al., 1999). However, studies primarily exploring the



**Figure 2** Hypermethylation of the *hMLH1* promoter region CpG island in early human gastric neoplasia. The presence of PCR product in lanes marked 'U' indicates unmethylated *hMLH1*; product in lanes marked 'M' indicates hypermethylated *hMLH1*. All primary tumors display unmethylated bands contributed by contaminating normal cells. Molecular size marker lane is at left. Both MSI-negative (33, 252, 59, 243, 163, 225, 207 and 218) and MSI-high (245) early gastric cancers are shown. Known hypermethylated SW48 cancer cells served as positive controls

frequency of MSI in intestinal metaplasia of the stomach remain to be performed. Thus, future studies of intestinal metaplasia for MSI and *hMLH1* hypermethylation may help resolve the dispute regarding which lesions comprise true gastric cancer precursors.

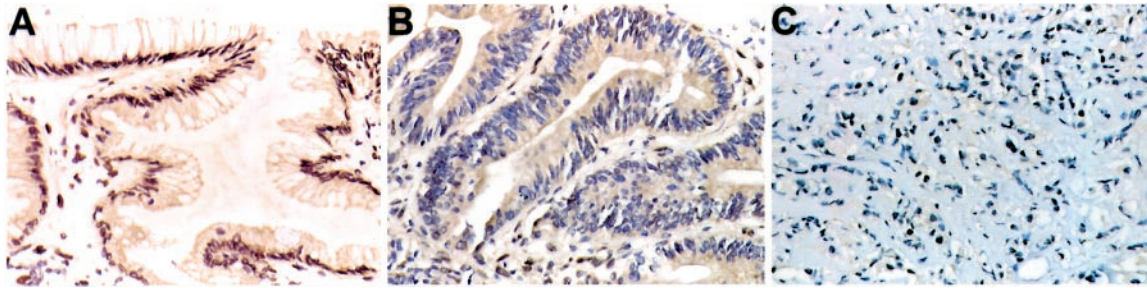
### Materials and methods

#### Patients

For the purposes of the current study, we defined early gastric neoplasia as encompassing both early gastric cancers and gastric adenomas. Early gastric cancer was defined as invasion limited to the mucosa and submucosa (but not the muscularis propria), whether or not regional lymph node metastases were present (Fukutomi et al., 1984; Eckardt et al., 1990). Sixty-four early gastric neoplasms were evaluated, comprising 28 adenomas, 18 mucosal carcinomas, and 18 carcinomas with superficial submucosal invasion but clear margins. All samples were obtained by endoscopic mucosectomy in Japan (G Tamura) after informed consent and under institutionally approved protocols. DNAs were extracted using standard techniques and were stored at 4°C. In addition, fragments of frozen tissue from some patients with carcinoma were stored at -80°C until use for immunohistochemical staining.

#### Microsatellite instability

The MSI status of 34 cancers was determined by PCR using fluoromer-labeled primers to amplify markers BAT25, BAT 26, D17S250, D5S346 and D2S123 (Boland et al., 1998). PCR was performed in three separate tubes for markers D17S250, BAT25 and Bat26. Markers D5S346 and D2S123 were multiplexed in the same reaction tube. Primers were fluorescently labeled as follows: BAT26 was labeled with a green dye, *tet*; BAT25 with a yellow dye, *hex*; and D5S346, D2S123 and D17S250 were all labeled with a blue dye, *fam*. PCRs were carried out in 10 µl volumes containing 60 ng genomic DNA, 1 pmol each primer, 50 mM KCl, 10 mM Tris, 0.4 mM each dNTP, 1.5 mM MgCl<sub>2</sub>, and 0.5 U Taq DNA Polymerase (Promega, Madison, WI, USA). PCRs consisted of an initial denaturation step at 94°C for 4 min, followed by 30 cycles at 94°C for 30 s, 55°C for 30 s, and 72°C for 30 s, and finally an additional extension step at 72°C for 6 min.



**Figure 3** *hMLH1* protein expression assessed by immunohistochemical staining in MSI-high early gastric cancers and matching normal mucosa. (A) High-power view of normal gastric mucosa, expressing abundant brown staining *hMLH1* protein in epithelial nuclei. (B, C) High-power view of gastric cancer with MSI and hypermethylation of *hMLH1*, showing diminished *hMLH1* protein with more basophilic (blue) staining of neoplastic nuclei

The annealing temperature varied among primer sets, but was usually between 50 and 60°C. Simultaneous gel electrophoresis of PCR products was performed on 5.25% Long Ranger gels (Bio-Rad, Hercules, CA, USA) containing 6 M urea. Samples were loaded onto 96-lane gels and electrophoresed on an ABI Prism automated DNA sequencer (Perkin Elmer, Norwalk, CT, USA). Gels were scored in two steps using separate programs. The first program, Genescan (ABI), checked for consistency across all lanes. This was also checked manually. The second program, Genotyper (ABI), assigned allele sizes and derived peak heights in arbitrary fluorescent units. Peak heights were then evaluated by hand, with the color and size of each peak identifying the product being evaluated and the presence of MSI. Since band profile was not always the same for each given primer set, it was very important to use Genescan and Genotyper to check for consistency and assign peak heights. In addition, four adenomas and one cancer were tested for MSI non-fluorometrically at loci D2S123, D2S147, D2S119, D11S904, and D17S250 using previously described methods (Parsonnet *et al.*, 1991; Suzuki *et al.*, 1994). Briefly, multiplex PCR with <sup>32</sup>P-labeled dCTP was performed with subsequent electrophoresis on polyacrylamide gels and visualization of products by autoradiography. Finally, 24 adenomas and one MSI-high cancer had been previously tested at 35 anonymous microsatellite loci (available on request).

Lesions were characterized as MSI-H if they manifested instability at two or more loci (or at more than 30% of loci if more than five loci were tested), MSI-L if unstable at only one locus (or less than 30% of loci if more than five loci were tested), and MSI-S if showing no instability at any locus (Boland *et al.*, 1998).

#### Methylation-specific PCR (MSP)

DNA methylation patterns in the *hMLH1* promoter were determined by MSP, as described previously (Herman *et al.*, 1996; Esteller *et al.*, 1998). MSP distinguishes unmethylated from hypermethylated alleles based on sequence alterations produced by 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, one  $\mu$ g of genomic DNA was denatured by treatment with NaOH and modified by sodium bisulfite. DNA samples were purified using Wizard DNA purification resin (Promega, Madison, WI, USA), 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 10 $\times$  PCR buffer (16.6 mM ammonium sulfate, 67 mM Tris, pH 8.8, 6.7 mM MgCl<sub>2</sub>, and 10 mM 2-mercaptoethanol), dNTPs (each at 1.25 mM), primers (300 ng each per reaction), and bisulfite-modified DNA (50 ng) in a final volume of 50  $\mu$ l. 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, UK) 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. DNA from colon cancer cell line SW48, which is completely hypermethylated at the *hMLH1* locus, was used as a positive control (Kane *et al.*, 1997). DNA from normal lymphocytes served as a negative control for hypermethylated *hMLH1*. Ten  $\mu$ l of each PCR reaction product were directly loaded onto non-denaturing 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), while 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).

#### Immunohistochemistry

Frozen tissue was thawed on ice, fixed in 10% formalin, and embedded in paraffin blocks. Five- $\mu$ m sections were mounted on glass slides. Paraffin-embedded tissues were deparaffinized with xylene for 30 minutes and rehydrated using graded ethanols. Antigen retrieval was performed using a heat-induced epitope retrieval method (Bankfalvi *et al.*, 1994). Immunoperoxidase staining using diaminobenzidine as chromogen was performed with the TechMate 1000 automatic staining system (Ventana, BioTek Solutions, Tucson, AZ, USA). Mouse monoclonal antibody (Ab) to the *hMLH1* gene product was used at 1:300 dilution (Pharmingen, San Diego, CA, USA). Staining of tumor cells was evaluated as present or absent in stained slides.

#### Statistical correlations

Analyses were performed using Statview 4.5 and super-ANOVA software for the Macintosh (SAS Institute Inc., Cary, NC, USA). Two-by-two table contingency analyses were performed using a two-tailed Fisher's exact test because some numerical values were less than five.

**Abbreviations**

Ab, antibody; hMLH1, human mut-L homologue 1; MMR, mismatch repair; MSI, microsatellite instability; MSP, methylation-specific PCR.

**Acknowledgments**

This work was partially supported by NIH grants CA85069, CA78843, DK 47717, and CA77057 (to SJ Meltzer), CA67497 (to SJ Meltzer and KT Wilson),

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DK53620 (to SP James, SJ Meltzer and KT Wilson), K08-DK02469 (to KT Wilson), DK09886-01 (to AS Fleisher), and the Office of Medical Research, Department of Veterans Affairs (SJ Meltzer and KT Wilson). JG Herman receives research funding and is entitled to sales royalties from INTERGEN, which is developing products related to research described in this paper. The terms of this arrangement have been reviewed and approved by The Johns Hopkins University in accordance with its conflict of interest policies. M Esteller is a recipient of a Spanish Ministerio de Educacion y Cultura Award.

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