Methylation of the *hMLH1* Promoter Correlates with Lack of Expression of hMLH1 in Sporadic Colon Tumors and Mismatch Repair-defective Human Tumor Cell Lines

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Methylation of the *hMLH1* Promoter Correlates with Lack of Expression of hMLH1 in Sporadic Colon Tumors and Mismatch Repair-defective Human Tumor Cell Lines[1]

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Abstract

Somatic mutations in DNA mismatch repair genes have been observed in sporadic tumors as well as cell lines and xenografts derived from such tumors implicating genetic defects of mismatch repair genes in the development of such tumors. However, the proportion of sporadic tumors in which mismatch repair genes have been inactivated has not been determined accurately. We have analyzed 66 sporadic colorectal tumors for the expression of hMLH1 by immunohistochemistry and identified 4 tumors that do not express hMLH1. These four colorectal tumors, a colon tumor cell line (SW48) and an endometrial tumor cell line (AN3CA), did not express hMLH1, despite the absence of mutations in its coding sequence. Cytosine methylation of the *hMLH1* promoter region was found in these four colorectal tumors, whereas cytosine methylation of the *hMLH1* promoter region was absent in adjacent normal tissue or in nine tumors that expressed hMLH1. In addition, cytosine methylation of the *hMLH1* promoter region was observed in the SW48 and AN3CA cell lines that do not express hMLH1 but not in four tumor cell lines known to express *hMLH1* mRNA. Our data indicate that DNA methylation is likely to be a common mode of mismatch repair gene inactivation in sporadic tumors.

Introduction

HNPCC[3] is a common cancer susceptibility syndrome characterized by cancers of the colon and numerous other sites and an early age of onset (1). A striking characteristic of HNPCC tumors is the presence of genetic instability most often detected as changes in the lengths of microsatellite sequences (2). Such MIN has been observed in varying proportion of numerous types of sporadic cancers, suggesting that the same types of genetic defects that underlie HNPCC can occur somatically in some sporadic cancers (reviewed in Ref. 3).

Studies performed over the last 3 years have indicated that a significant proportion of HNPCC is due to inherited defects in DNA mismatch repair genes (reviewed in Refs. 4–6). Biochemical and genetic studies in eukaryotes have defined five genes, *MSH2, MSH3, MSH6* (also called *GTBP*), *MLH1,* and *PMS2* (called *PMS1* in *Saccharomyces cerevisiae*), encoding proteins that are homologues of bacterial mismatch repair proteins and are required for eukaryotic DNA mismatch repair (reviewed in Refs. 5 and 7). A sixth human gene, *PMS1,* has also been suggested to be important for mismatch repair, although biochemical studies supporting such a relationship are not yet available (reviewed in Ref. 5). Approximately 70% of MIN+ HNPCC kindreds can be ascribed to mutations in DNA mismatch repair genes; the majority of germ-line mutations have been found in *hMSH2* and *hMLH1,* whereas germ-line mutations in *hPMS1* and *hPMS2* appear to be much rarer, and germ-line mutations in *hMSH3* and *hMSH6* have not yet been reported (8). Somatic mutations in *hMSH2* and *hMLH1* have been reported in some MIN+ sporadic cancers and MIN+ familial colon cancers not meeting Amsterdam Criteria for HNPCC, suggesting the involvement of mutation of DNA mismatch repair genes in sporadic cancers (9–12). However, the proportion and spectrum of sporadic cancers associated with mutations in mismatch repair genes has not been clearly elucidated at this time.

The identification of human DNA mismatch repair genes has made it possible to investigate the relationship between genetic defects in mismatch repair genes and inherited and sporadic cancers. Such studies have been problematic for two reasons: (*a*) the methods available for detecting mutations in these genes are tedious, and no single method is capable of detecting all of the types of mutations that could exist. This is particularly problematic when tumor samples are analyzed because of the contamination of tumor cells with normal tissue in such samples; and (*b*) the complete spectrum of mutations and modes of inactivation applicable to the analysis of these genes is not presently known. We have examined the expression of hMLH1 in sporadic colon tumors and found that lack of expression of hMLH1 in 4 of 66 sporadic colon tumors and both a colon and an endometrial tumor cell line correlates with methylation of the *hMLH1* promoter region. These results indicate that DNA methylation is likely to be a common mode of mismatch repair gene inactivation in sporadic tumors.

Patients and Methods

Patients. Sixty-six sporadic adenocarcinomas were studied. Patients were operated on at the Deaconess Hospital between 1989 and 1993. Formalin-fixed, paraffin-embedded tissue blocks containing both tumor and adjacent normal colonic epithelium were available for each case. Frozen tumor samples were also obtained for 13 of the 66 samples for more extensive analysis.

Immunohistochemistry. Five-μm sections were mounted on Superfrost Plus glass slides and baked overnight at 45°C. Slides were deparaffinized in xylene, re-hydrated in graded alcohols, and washed in water. Antigen retrieval was accomplished by microwave irradiation; slides were placed in a pressure cooker filled with antigen retrieval Citra solution (Biogenex, San Ramon, CA), which was placed in a microwave oven at 750 W for 30 min. Slides were allowed to cool for 30 min at room temperature prior to staining. Immunohistochemistry was carried out in a Ventana 320/ES automated immunohistochemistry instrument (Ventana Medical Systems, Tuscon, AZ) as described previously (13). Monoclonal anti-hMLH1 antibody (clone 14, 3 mg/ml; Oncogene Science, Cambridge, MA) was diluted at 1:50 and incubated for 20 min. A posttreatment was step employed using a glutaraldehyde fixative (0.05% glutaraldehyde, 0.9% sodium chloride in distilled water) for 8 min. Normal tissue adjacent to tumor was used as internal positive control. For

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[3] The abbreviations used are: HNPCC, hereditary nonpolyposis colorectal carcinoma; MIN, microsatellite instability.

negative controls, a nonspecific primary mouse monoclonal antibody (clone MOPC-21), which is directed against an epitope not found in human tissue, was used (14). Antigen-antibody reactions were revealed with standardized development times by the instrument using the avidin-horseradish peroxidase and diaminobenzidine as substrate. Slides were counterstained with methyl green and coverslipped. Staining of hMLH1 in both tumor and normal tissue was scored independently by two pathologists (J. L. and M. L.) on an intensity scale from 0 to 3, without knowledge of clinical, pathological, or methylation status data.

DNA Samples. Snap-frozen tumor and normal tissue from 13 cases was retrieved from the Colorectal Frozen Tissue Tumor Bank. OCT-embedded tissue immediately adjacent to the snap-frozen material was cut, stained with H&E, and verified to represent normal and tumor tissue. For DNA extraction, samples were weighed, minced using a tissue homogenizer, and placed in digestion buffer [50 mM Tris-HCl buffer (pH 8.0), 1 mM EDTA, and 0.5% Tween 20] containing 200 μg/ml Proteinase-K (Life Technologies, Inc., Gaithersburg, MD) for 24 h at 37°C. Following phenol-chloroform purification and ethanol precipitation, pellets were washed with 70% ethanol, lyophilized, and resuspended in sterile water. Spectrophotometric measurements were made to record DNA yield and purity.

Extraction of genomic DNA from tumor cell lines has been described previously (15). The cell lines analyzed were EA-1 (endometrial: *hMSH2* mutant, hMLH1 expressed), DU145 (prostate: *hMLH1* splice site mutant that expresses mutant *hMLH1* mRNA), MKN45 (gastric: mismatch repair proficient), SW48 (colon: no *hMLH1* mRNA expressed), AN3CA (endometrial: no *hMLH1* mRNA expressed), and LOVO (colon: *hMSH2* deletion mutant, hMLH1 expressed).

DNA Sequencing and Mutation Detection. DNA samples were analyzed for the presence of mutations in *hMLH1* by direct DNA sequencing as described previously (16). Briefly, individual *hMLH1* exons were amplified by PCR, and the product DNA was sequenced with dye primer chemistry on ABI 373 or ABI 377 automated sequencers. DNA sequence changes were detected by comparison with the wild-type *hMLH1* sequence using Sequencher 3.0 software (GeneCodes, Ann Arbor, MI), and heterozygous nucleotides were detected by visual inspection of the sequencing chromatograms. The *hMLH1* upstream region was sequenced from P1 clone 1263 on an ABI 373 DNA sequencer using dye terminator chemistry and primers derived from the DNA sequence essentially as described (16). Contigs were assembled using Sequencher 3.0 software, and searches for transcription factor consensus binding sites were performed using MacVector software.

Promoter Methylation Assays. Genomic DNA samples were digested with restriction endonucleases in 20-μl volumes of restriction endonuclease buffer (as supplied by New England Biolabs, Beverly, MA) containing 250 ng of genomic DNA and 0.004 pg of pRDK447 DNA (a 9.4-kb plasmid containing the *yMSH2* gene). pRDK447 DNA served as an internal control for cleavage by *Hpa*II and *Msp*I because it could be cleaved to completion by these enzymes and because the CpG sites in the recognition sequences of these enzymes are not cytosine methylated during propagation in *Escherichia coli*. Reactions contained either no enzyme, 75 units of *Hpa*II (New England Biolabs), or 150 units of *Msp*I (New England Biolabs) for 6 h at 37°C.

To analyze cleavage of the *hMLH1* promoter region, 12.5 ng of DNA from each digest was analyzed by PCR in 25-μl reactions containing 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 1.5 mM $MgCl_2$, 50 μM each of the four deoxynucleotide triphosphates, 0.75 units of AmpliTaq DNA polymerase (Perkin-Elmer, Norwalk, CT), and 2.5 pmol of each primer 27494 (5′-CGCTCGTAGTATTCGTGC) and 25266 (5′-TCAGTGCCTCGTGCTCAC) designed to amplify nucleotides −670 to −67 of hMLH1. PCR was performed for one cycle of 95°C for 5 min followed by 33 cycles of 94°C for 30 s, 55°C for 30 s, and 72°C for 30 s, followed by one cycle of 72°C for 7 min. The resulting amplification products were then analyzed by agarose gel electrophoresis using standard methods. Analysis of DNA methylation was performed in the absence of knowledge of the hMLH1 expression status in all experiments.

Cleavage of the control DNA was analyzed essentially as described above for the *hMLH1* promoter region, with the following minor modifications. The reactions contained 2.5 pmol of each primer 27859 (5′-TTCTTGGAGGACGACAGC) and 27860 (5′-CAATCACATCTAAATGCG), which amplify a 567-bp piece of *yMSH2* that contains a *Hpa*II site in the middle in place of the *hMLH1* primers. In addition, the number of amplification cycles was increased to from 35 to 38 in a number of individual experiments, and different annealing temperatures ranging from 55°C to 57°C were examined.

Results

Sequence of the *hMLH1* Upstream Region. To begin to understand the structure of the *hMLH1* promoter region, the sequence of nucleotides −1 to −1554 upstream of the ATG of *hMLH1* was determined by standard sequence analysis of a P1 phage containing the NH_2 terminus of *hMLH1* (Genbank accession number U83845). This sequence confirms and extends another sequence present in Genbank (accession number U26559). The sequence from −1 to −1295 was analyzed for the presence of transcription factor consensus binding site sequences, and this analysis revealed the presence of numerous such sites (Table 1). At present, we have no definitive data indicating which, if any, of these sites functions in the transcription of this gene. However, their presence suggests that this region of *hMLH1* is important for regulation of its transcription. The entire upstream region from nucleotides −1295 to −1 was 56.4% G+C. The region between nucleotides −670 to −67 of the promoter region that was amplified by PCR for analysis of methylation status was 58.4% G+C and had 7.3% CpG sites that conceivably could serve as cytosine methylase substrates. Importantly for the analysis described in subsequent sections, *Hpa*II recognition sites were found at nucleotide positions −567, −527, −347, and −341.

Methylation of the *hMLH1* Promoter Region in Cell Lines That Do Not Express hMLH1. Two different cell lines, the colon tumor cell line SW48 and the endometrial tumor cell line AN3CA, have been shown not to express *hMLH1* mRNA (10, 15). Furthermore, extracts prepared from these cell lines are mismatch repair defective *in vitro* and show *in vitro* complementation properties consistent with lack of expression of hMLH1 (15). The entire coding sequence and intron exon regions of the *hMLH1* gene was sequenced from genomic DNA isolated from these cell lines, and no *hMLH1* mutation was observed (data not shown). These data suggest that the absence of *hMLH1* mRNA in these cell lines was secondary to lack of transcription of the *hMLH1* gene rather than to the presence of a mutation that affected either translation or splicing of the *hMLH1* mRNA.

The *hMLH1* promoter region in SW48 and AN3CA was examined using a PCR assay (Fig. 1). Included in this analysis were four control cell lines known to either be proficient for mismatch repair (MKN45), to express a mutant *hMLH1* mRNA (DU145), or be mismatch repair deficient due to the presence of a *hMSH2* mutation but express wild-type hMLH1 protein (EA-1 and LOVO; Refs. 10 and 15). The results of this analysis showed that the *hMLH1* promoter region from SW48 and AN3CA was resistant to digestion by *Hpa*II and sensitive to digestion by *Msp*I. In all cases, the promoter region of the control cell lines was sensitive to digestion by *Hpa*II and *Msp*I. In all experiments, the unmethylated internal control DNA was sensitive to

Table 1 *Transcription factor binding site consensus sequences present in the 5′ region of hMLH1*

Nucleotides −1295 to 116 of the *hMLH1* gene were searched for transcription factor binding site consensus sequences as described in "Material and Methods." Those consensus sequences identified upstream of nucleotide 1 are listed.

AP1	E2A/bHLH	Pur-1
AP2	E2FEKLF	Pur-1.S
ARE	ETS	Pu box
ATF	GATA	RARE
C/EBP	GRE	Sp1
CAAT (CF1)	HNF-6	SREth1/E47
CAAT (CP2)	Homeo	TTF-1
COUP/EAR	NF-kB	USF
CP1/NFY	NFR2 GABP	VIT-D
CREB/ATF	OCT-1 & 2	XRE
E2 box	p53	ZID

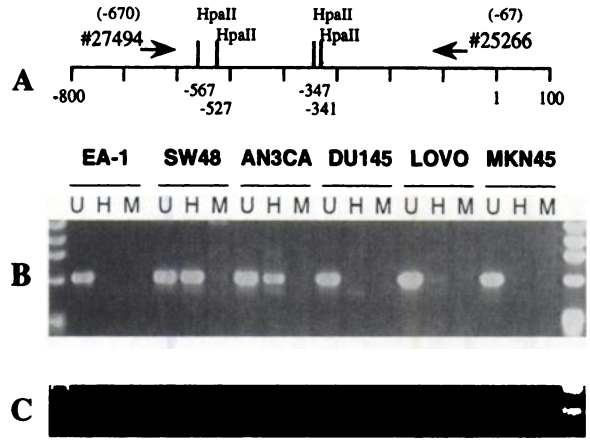

Fig. 1. Analysis of human tumor cell lines for methylation of the *hMLH1* promoter region. *A*, map of the *hMLH1* promoter region. The positions of the *Hpa*II sites and PCR primers used in the analysis of DNA methylation status are indicated. *B*, amplification of the *hMLH1* promoter region from the indicated cell line DNAs before or after digestion with the indicated restriction endonucleases. *C*, amplification of unmethylated internal control DNA before or after digestion with the indicated restriction endonucleases. *U*, undigested; *H*, digested with *Hpa*II; *M*, digested with *Msp*I.

digestion by *Hpa*II and *Msp*I. These data are consistent with methylation at CpG sites at all four *Hpa*II sites between −670 and −67 of the *hMLH1* promoter region in the SW48 and AN3CA cell lines that do not express *hMLH1* mRNA.

A Subset of Sporadic Colon Tumors Do Not Express hMLH1 Protein. Previous studies have used immunohistochemical analysis to identify MIN+ endometrial tumors that do not express hMSH2 protein and MIN+ colon tumors that do not express hMSH2 or hMLH1 proteins (11, 17). A series of 66 sporadic colon tumors were analyzed by staining paraffin sections with anti-hMLH1 monoclonal antibodies (Fig. 2). In all cases, the adjacent normal tissue showed nuclear staining with particularly intense staining of the crypts. In three of the cases, tumor tissue did not show any staining, consistent with lack of hMLH1 expression (also see Fig. 3, cases 1, 4, and 5), and in one case, while the majority of cells did not express hMLH1, there were subpopulations of cells expressing hMLH1, presumably representing different clones. In the remaining 62 cases, the tumor tissue and normal tissue showed similar levels of staining consistent with expression of the hMLH1 protein.

Lack of hMLH1 Expression Correlates with Methylation of the *hMLH1* Promoter Region. In an initial survey, three of the hMLH1 non-expressing tumors and two of the hMLH1-expressing tumors were examined for the presence of *hMLH1* mutations. No mutation in *hMLH1* was found in genomic DNA isolated from the normal or tumor tissue from these cases by sequencing the entire coding and exon-intron sequence of *hMLH1* (data not shown). In one case, the common A to G Ile217Val polymorphism was found in both the normal and tumor tissue, and in two cases, an A to G polymorphism was found at nucleotide −19 of the splice acceptor site of intron 14.

The *hMLH1* promoter region present in genomic DNA isolated from the normal and tumor tissue from these cases was analyzed for DNA methylation essentially as described for the cell lines discussed above (Fig. 3). In the case of the three colon tumors that did not express hMLH1 protein (Fig. 3, cases 1, 4, and 5), the DNA isolated from the tumor tissue was highly resistant to digestion by *Hpa*II and sensitive to digestion by *Msp*I, whereas DNA isolated from the normal tissue was sensitive to digestion by both *Hpa*II and *Msp*I. This was in contrast to the two colon tumors that expressed hMLH1 protein. In one case (Fig. 3, case 3), the DNA isolated from both the tumor and normal tissue was sensitive to digestion by both *Hpa*II and *Msp*I. In the other case (Fig. 3, case 2), the DNA isolated from both the tumor and normal tissue was sensitive to digestion by both *Hpa*II and *Msp*I, although a small amount of *Hpa*II-resistant DNA was seen in the tumor DNA; possibly this tumor contained some cells that did not express hMLH1, or possibly one allele of hMLH1 was methylated. Subsequent to this analysis, one additional hMLH1 non-expressing tumor (with rare tumor cell nests expressing hMLH1) and seven hMLH1-expressing tumors were examined for methylation of the *hMLH1* promoter region (data not shown; none of these cases were tested for the presence of *hMLH1* mutations). Partial *Hpa*II resistance of the *hMLH1* promoter was found in the tumor that did not express hMLH1, consistent with the tumor tissue being a mixture of cells that expressed and did not express hMLH1. In contrast, methylation was

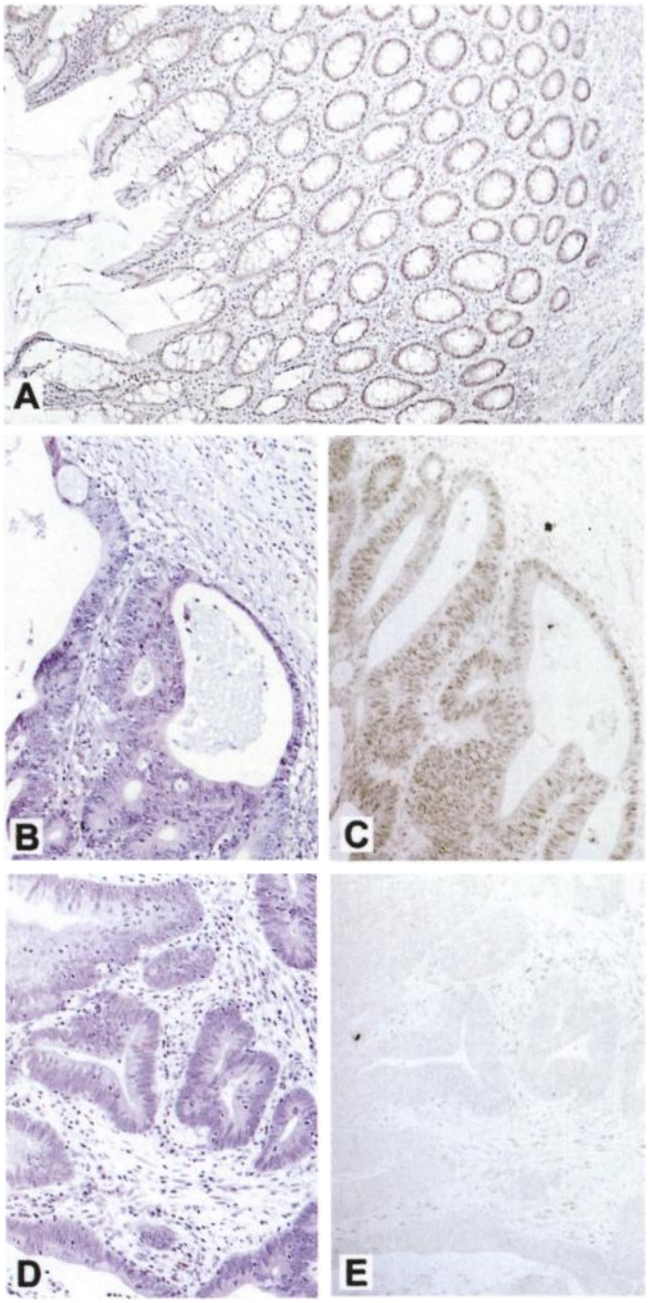

Fig. 2. Immunohistochemical analysis of hMLH1 expression in normal colonic tissue and colon adenocarcinoma. *A*, normal colon tissue stained with anti-hMLH1 antibody showing positive nuclear staining for hMLH1, particularly in the crypts. *B*, adenocarcinoma (H&E staining). *C*, adjacent section to that in *B* stained with anti-hMLH1 antibody showing positive nuclear staining for hMLH1. *D*, adenocarcinoma (H&E staining). *E*, adjacent section to that in *D* stained with anti-hMLH1 antibody showing no hMLH1 expression.

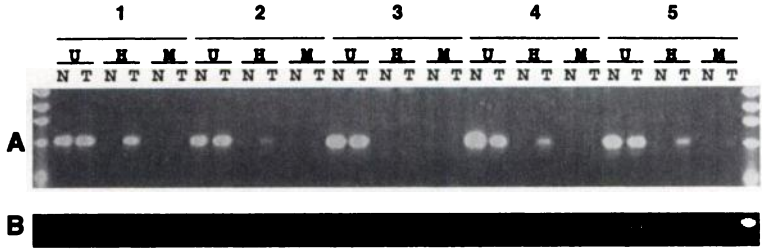

Fig. 3. Analysis of human colon tumors for methylation of the hMLH1 promoter region. *A*, amplification of the hMLH1 promoter region from the indicated tumor (*T*) or adjacent normal (*N*) DNAs before or after digestion with the indicated restriction endonucleases. *B*, amplification of unmethylated internal control DNA before or after digestion with the indicated restriction endonucleases. Cases 1, 4, and 5 did not express hMLH1, and cases 2 and 3 expressed hMLH1 by immunohistochemistry analysis. *U*, undigested; *H*, digested with *Hpa*II; *M*, digested with *Msp*I.

not observed in the seven cases that expressed hMLH1 (data not shown, but similar to Fig. 3, case 3). In all experiments, the unmethylated internal control DNA was sensitive to digestion by *Hpa*II and *Msp*I. These data are consistent with methylation at CpG sites at all four *Hpa*II sites between −670 and −67 of the *hMLH1* promoter region in the tumors that do not express *hMLH1* mRNA.

Discussion

Considerable evidence exists indicating that the MIN+ phenotype observed in HNPCC tumors is due to inactivation of DNA mismatch repair in the tumors resulting from inherited mutations in DNA mismatch repair genes (reviewed in Refs. 3–6). The observation that many sporadic tumors also show a MIN+ phenotype has suggested that such tumors may also be mismatch repair defective due to somatic mutations in the same DNA mismatch repair genes implicated in HNPCC (Refs. 9–12); reviewed in Refs. 3–6). Although somatic mutations in *hMSH2* and *hMLH1* have been demonstrated in some MIN+ sporadic tumors, mutations in mismatch repair genes have clearly not been observed in all MIN+ sporadic tumors (9–12). The data presented here demonstrate the existence of a class of sporadic colon tumors, a colon tumor cell line, and an endometrial tumor cell line that do not express hMLH1 and do not have mutations in the *hMLH1* coding sequence. In these, lack of hMLH1 expression correlated with cytosine methylation of the *hMLH1* promoter region. These observations suggest that inactivation of gene expression by DNA methylation is likely to be an important mechanism of inactivation of DNA mismatch repair genes in sporadic tumors similar to that seen for other genes, the inactivation of which has been demonstrated in tumors (18–20). More extensive studies will be required to determine the proportion and spectrum of tumors in which expression of mismatch repair genes has been inactivated.

The results of the studies presented here have important implications for the analysis of the role that DNA mismatch repair defects play in sporadic cancers. Clearly, mutations in the coding sequence of mismatch repair genes are not the only route to inactivation of DNA mismatch repair. Thus, it will be important to consider inactivation of *hMLH1* and possibly other mismatch repair genes by DNA methylation in studies the goal of which is to determine the proportion and spectrum of sporadic tumors containing mismatch repair defects. Our studies and those of others (11, 17) have demonstrated that immunohistochemistry is likely to be a convenient and widely available method for use in screening tumor samples for lack of expression of mismatch repair genes. The DNA methylation assay described here and other such assays (18–20) should also prove to be useful as a screening tool for evaluating the expression status of *hMLH1* and possibly other mismatch repair genes and the mode of inactivation of these genes. This assay must be performed in a carefully controlled manner and will be most reliable when used in conjunction with other methods of monitoring gene expression such as immunohistochemistry, as used here.

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