

## Detection of Aberrantly Methylated *hMLH1* Promoter DNA in the Serum of Patients with Microsatellite Unstable Colon Cancer<sup>1</sup>

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### Abstract

Serological tumor markers have proven valuable in the care of individuals with cancer for the early detection of primary cancers, early detection of cancer relapse, monitoring the response of cancers to therapy, and as predictors of cancer prognosis. Recently, the aberrant hypermethylation of the *hMLH1* promoter and its consequent transcriptional silencing has been shown to be a common event in the formation of sporadic microsatellite unstable colon cancer. The silencing of *hMLH1* expression appears to be controlled by the hypermethylation of a specific region in the *hMLH1* promoter. We developed a methylation-specific PCR assay that assesses this region of the *hMLH1* promoter. We found that this assay is able to detect methylated *hMLH1* promoter DNA in the serum of some patients with microsatellite unstable colon cancers. In a panel of sera from 19 colon cancer cases, 9 with *hMLH1* promoter methylation in the tumor primary, the assay proved 33% sensitive and 100% specific. This assay offers a potential means for the serum-based detection and/or monitoring of microsatellite unstable colon cancers.

### Introduction

Colorectal cancer is the second most common cause of cancer mortality in the adult American population (1). Approximately 40% of affected individuals will ultimately die from their cancer. The chance of cure is greatest in those individuals whose tumor primary or tumor relapse is detected at an early stage that permits curative surgery. Early detection is particularly challenging for tumors that arise in the proximal colon, which is farthest from the rectum and requires full colonoscopy for direct inspection. One novel approach to colon cancer detection has been the attempt to detect tumor-specific DNA alterations in DNA extracted from the blood of patients with cancer (2). In particular, circulating colon cancer DNA has been identified by detection in serum of *p53* and *K-RAS* mutations, genetic alterations that are common in tumors that arise in the distal colon. However, tumors of the proximal colon commonly arise via the pathway of MSI<sup>3</sup> in which *p53* and *K-RAS* are most commonly wild type (3, 4). Previous efforts attempting to detect the signature of MSI in DNA extracted from the serum of individuals bearing tumors of this class have been unsuccessful, suggesting a need for alternative approaches (2).

Colon cancers with MSI are initiated by inactivation of genes that encode components of the DNA mismatch repair pathway. Germ-line mutation of any member of this group of genes gives rise to a syndrome of familial MSI colon cancers, known as hereditary non-polyposis colon cancer (5, 6). However, most commonly, MSI colon

cancers arise sporadically in individuals with no familial colon cancer history. In these cases, the MSI pathway most commonly is initiated by silencing of the *hMLH1* DNA repair gene via aberrant methylation of the *hMLH1* promoter (7–9). The methylated *hMLH1* promoter DNA is thus a tumor-specific modification that potentially could serve as a target for detection by serological assays. Because the reaction of DNA with sodium bisulfite chemically converts cytosines to uracil but does not alter methyl-cytosines, PCR primers can be designed that, following bisulfite treatment, will specifically amplify methylated but not nonmethylated target DNA templates (10). The present study was designed to determine the ability of such a methylation-specific PCR assay to detect methylated *hMLH1* promoter DNA as a serum marker of MSI colon cancer in individuals with this disease.

### Materials and Methods

**Sample Collection and DNA Preparation.** Primary colon cancer samples and preoperative serum samples were obtained from 19 patients. The tumor samples were immediately snap frozen and prepared as described previously (11). Serum samples were prepared immediately after collection by centrifuging the sample at  $2700 \times g$  for 15 min and aspirating the serum. The serum was then stored at  $-80^{\circ}\text{C}$  until use. DNA was extracted from the serum by incubating 1 ml of serum in a buffered extraction solution (140 mM Tris, 140 mM EDTA, and 0.57% SDS) with proteinase K (500  $\mu\text{g}$ ) overnight at  $50^{\circ}\text{C}$  in a shaking water bath. The sample was then subject to phenol:chloroform/chloroform DNA extraction twice, chloroform extraction, and ethanol precipitation. The precipitated DNA was resuspended in 25  $\mu\text{l}$  of distilled, deionized water and stored at  $-20^{\circ}\text{C}$ . Approximately half of the sample was used in the subsequent MSP assay.

**Cell Lines and Primary Tumors.** The cell lines RKO, SW480, Vaco-5, and Vaco-457 were used as known controls in the studies described below. The establishment and maintenance of the Vaco colon cancer cell lines has been described previously (11, 12). Genomic DNA from the cell lines and tumors was extracted using protocols published previously (11).

**MSP of *hMLH1*.** MSP exploits the effect of sodium bisulfite on DNA, which efficiently converts unmethylated cytosine to uracil but which leaves methylated cytosine unchanged. Consequently, after treatment the methylated and unmethylated alleles have different sequences that can be used to design allele-specific primers.

Genomic DNA was modified with sodium bisulfite as has been described previously (10, 13). Briefly, genomic DNA from the sample (1  $\mu\text{g}$ ) was diluted in 50  $\mu\text{l}$  of distilled water and then denatured by 0.2 M sodium hydroxide for 15 min at  $37^{\circ}\text{C}$ . Thirty  $\mu\text{l}$  of 10 mM hydroquinone (Sigma) and 520  $\mu\text{l}$  of 3 M sodium bisulfite (Sigma) at pH 5.0 were then added, and the samples were incubated at  $53^{\circ}\text{C}$  for 18–20 h. After treatment, the DNA was purified using the Wizard DNA Clean-Up System (Promega), following the manufacturer's protocol. The DNA was then desulfonated with 0.3 M sodium hydroxide for 10 min at room temperature, neutralized with 17  $\mu\text{l}$  of 10 M  $\text{NH}_4\text{OAc}$ , and then precipitated in 100% ethanol overnight at  $-80^{\circ}\text{C}$ . The samples were resuspended in distilled water at a final concentration of  $\sim 50$  ng/ $\mu\text{l}$  and stored at  $-80^{\circ}\text{C}$  for up to 8 weeks. A set of four known methylated and unmethylated control DNA samples were included in each round of bisulfite treatment.

The modified DNA was then subject to MSP using primer pairs engineered to amplify either methylated or unmethylated DNA. The primers anneal to *hMLH1* promoter sequence that contains a total of five CpG dinucleotides,

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<sup>3</sup> The abbreviations used are: MSI, microsatellite instability; MSS, microsatellite stable; MSP, methylation-specific PCR.

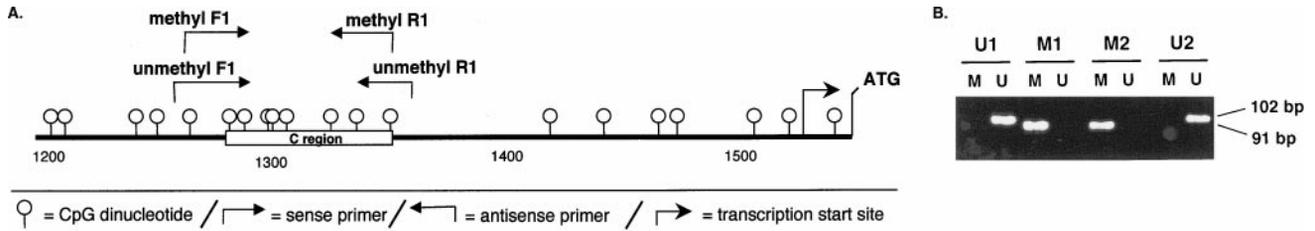


Fig. 1. A, location of primers for *hMLH1* MSP assay in relation to the *hMLH1* promoter C region identified by Deng *et al.* (14). Primers cover 5 CpG dinucleotides in this region and are designed to specifically amplify sodium bisulfite-converted sequences arising from either methylated (*methyl*) or unmethylated (*unmethyl*) templates. The numbering scheme is based on the GenBank sequence entry U83845. B, results of *hMLH1* MSP assay using primers that amplify methylated (M) or unmethylated (U) alleles specifically. M1 and M2 are DNA samples from cell lines V5 and RKO, respectively, shown previously to have only methylated *hMLH1* alleles, whereas U1 and U2 are DNA samples from cell lines SW480 and V457, respectively, that bear only unmethylated *hMLH1* alleles (9, 14).

with at least one CpG dinucleotide placed at the 3' end of each primer to maximize discrimination between methylated and unmethylated alleles after sodium bisulfite treatment (Fig. 1A). Furthermore, the primers were designed to recognize the "C" region in the *hMLH1* promoter, the methylation status of which has been demonstrated to invariably correlate with *hMLH1* expression in colon cancer (14). The methylated and unmethylated primer pair sequences were as follows: methyl specific, 5'-AACGAATTAATAGGAAGAGCGGAT-AGCG-3' (sense) and 5'-CGTCCTCCCTAAAACGACTACTACCC-3' (antisense); and unmethyl specific, 5'-TAAAAATGAATTAATAGGAAGAGTG-GATAGTG-3' (sense) and 5'-AATCTCTTCATC CCTCCCTAAAACA-3' (antisense). The primer pairs were purchased from Sigma-Genosys (The Woodlands, TX).

The PCR was performed in 20- $\mu$ l reaction volumes containing 1 $\times$  PCR buffer II (Perkin-Elmer), 1.5 mM MgCl<sub>2</sub>, 200  $\mu$ M deoxynucleotide triphosphates mixture (Perkin-Elmer), 1  $\mu$ M of each primer, 100–150 ng of modified DNA, and 1.5 units of Amplitaq gold DNA polymerase (Perkin-Elmer). The thermocycler conditions were as follows: methyl specific, 95°C for 10 min, (92°C for 30 s, 62°C for 30 s, 70°C for 30 s) for 39 cycles, and final extension of 70°C for 7 min; and unmethyl specific, 95°C for 10 min, (92°C for 30 s, 57°C for 30 s, 70°C for 30 s) for 39 cycles, and final extension of 70°C for 7 min. The PCR products were subject to gel electrophoresis through a 2.5% agarose gel, stained with ethidium bromide, and then visualized with UV illumination using a digital imaging system (Alpha Inotech).

## Results and Discussion

To attain maximal sensitivity and specificity, primers for *hMLH1* MSP were designed that, following bisulfite conversion, would selectively amplify templates derived from either methylated or unmethylated versions of sequences contained within the "C" region of the *hMLH1* promoter (Ref. 14; Fig. 1A). Methylation of the "C" region, which corresponds to bp -248 to -178 relative to the transcription start, has been demonstrated to invariably correlate with *hMLH1* expression in colon cancer (14). To further enhance the discrimination between products amplified by the primer sets that amplified methylated *versus* unmethylated *hMLH1*-derived templates, the primers were designed to generate PCR products that varied in size by 11 bp (102 bp *versus* 91 bp). The short size of these PCR products also increases the utility of these primers for determining the methylation status of the *hMLH1* promoter in samples of paraffin-embedded, formalin-fixed tumor tissues.

These primers were first tested for their specificity in amplifying methylated or unmethylated *hMLH1* promoter DNA by assaying four cell lines that previously had been characterized as, respectively, demonstrating an active or a methylated and silenced *hMLH1* promoter. As shown in Fig. 1B, a PCR product was obtained with the methylation-specific primer only from those cell lines shown previously by other assays to have a silenced and methylated *hMLH1* promoter (8, 9).

To determine the sensitivity of this methylation-specific PCR assay for detecting methylated *hMLH1*, DNA from cell lines with methylated *hMLH1* alleles was mixed with DNA from cell lines bearing

unmethylated *hMLH1* alleles. These mixtures were treated with sodium bisulfite and then subjected to the MSP assay. A minimum of 1.0% of methylated DNA could be detected with this assay. On the basis of the amount of input DNA used in these reactions, the assay successfully detected the methylated *hMLH1* promoter in 1.5 ng of total genomic DNA, which is equivalent to the amount of DNA present in 100–200 cells.

This assay was then used to determine the presence or absence of *hMLH1* promoter methylation in a collection of 19 colon cancers that we had previously characterized for their MSI status (data not shown) and for which matched serum samples had been obtained prior to the surgical resection of the cancer. Of 10 MSI colon cancers, 90% ( $n = 9$  of 10) demonstrated methylation of the *hMLH1* promoter. In contrast, 0 of 9 MSS colon cancer demonstrated *hMLH1* promoter methylation (see Table 1 and Fig. 2).

Table 1 Results of *hMLH1* MSP in tumors and serum of colon cancer patients  
Description of patient and tumor characteristics compared with results of *hMLH1* MS-PCR analysis of matched tumor and serum samples.

Patient no.	<i>hMLH1</i> Tumor DNA/Serum DNA <sup>a</sup>	MSI status	Stage	Grade
1	M/M	MSI	D	Poor
2	M/M	MSI	D	Moderate
3	M/M	MSI	C	Poor
4	M/U	MSI	C	Moderate
5	M/U	MSI	C	Moderate
6	M/U	MSI	B2	Moderate
7	M/U	MSI	B	Moderate
8	M/U	MSI	B	Moderate
9	M/U	MSI	A	Moderate
10	U/U	MSS	D	Moderate
11	U/U	MSS	D	Moderate
12	U/U	MSS	D	Poor
13	U/U	MSS	C	Moderate
14	U/U	MSS	B	Moderate
15	U/U	MSS	B	Moderate
16	U/U	MSS	B	Moderate
17	U/U	MSI	B	Moderate
18	U/U	MSS	B	Moderate
19	U/U	MSS	HGD <sup>b</sup>	HGD

<sup>a</sup> M, methylated *hMLH1* promoter; U, unmethylated *hMLH1* promoter.

<sup>b</sup> HGD, high-grade dysplasia in adenoma.

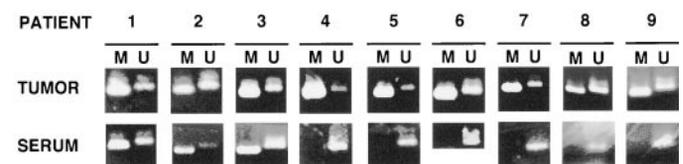


Fig. 2. Results of *hMLH1* MSP from colon cancers and paired serum samples. M and U, assays specific for amplification of the methylated *versus* unmethylated, respectively, *hMLH1* promoter. The unmethylated alleles detected in tumor samples likely represent contaminating normal tissue elements and in serum samples likely arise from DNA released from circulating leukocytes.

Using the *hMLH1* promoter MSP assay, we then tested the DNAs extracted from the preoperative serum samples that matched these same 19 colon cancers. Of nine individuals whose MSI colon cancers tested positive for *hMLH1* promoter methylation, 33% ( $n =$  three of nine) also demonstrated a positive assay for *hMLH1* promoter methylation in DNA from their preoperative serum samples (see Table 1 and Fig. 2). A positive serum result was obtained in two of two individuals found at surgery to have distant metastases (stage D); in one of three individuals found to have nodal metastases (stage C); and in none of four individuals whose tumors proved to be confined to the colon (stage B). None of 10 preoperative serum samples tested positive in the cases of individuals whose tumors lacked *hMLH1* promoter methylation (Table 1). Individuals whose tumors lacked *hMLH1* promoter methylation were similar to those whose tumors demonstrated *hMLH1* promoter methylation with regard to stage, location of the primary tumors, and age of the patients at diagnosis. Thus in this sample set, the assay of serum DNA for *hMLH1* promoter methylation demonstrated a sensitivity of 33% and a specificity of 100% for the detection of colon cancers bearing this molecular alteration.

The detection of methylated *hMLH1* promoter DNA in the serum of a group of patients with MSI colon cancers contrasts with the previous inability to detect microsatellite alterations in serum-DNA samples from a similar group of MSI colon cancer patients (2). This difference likely reflects the fact that PCR amplification of the methylation-specific *hMLH1* promoter product from bisulfite-treated DNA eliminates the background signal from normal DNAs. In contrast, sensitivity for detecting in serum the characteristic microsatellite shifts of the DNA from MSI tumors is likely compromised by a considerable background of the normal microsatellite alleles that would also be expected to be amplified from normal DNA also present in serum. Because MSI cancers are in general wild type for *p53* and *K-RAS*, the serological detection of methylated *hMLH1* promoter DNA provides a potential unique serological marker for this subset of cancers.

The sensitivity of 33% that we find for the detection of methylated *hMLH1* promoter DNA in serum is similar to the sensitivity reported for detecting methylated *p16* promoter DNA in the serum of patients with non-small cell lung cancer, head and neck cancer, and breast cancer (15–17) but is less than the sensitivity reported for the detection of methylated *p16* DNA in the serum of patients with hepatocellular carcinomas that carry methylated *p16* (80%) and less than the sensitivity reported for detection of *p53* mutations in the serum of patients with *p53* mutant colon cancers (70%; Refs. 2 and 18). These differences suggest that future technical advances may permit additional improvement in the sensitivity of serological detection of methylated *hMLH1* promoter DNA. This would potentially allow application of the assay for early detection of *hMLH1* methylated colon cancers, which usually occur in the proximal (right-sided) colon and so are occult to early detection by such standard screening modalities as flexible sigmoidoscopy. Detection of *hMLH1* promoter methylation in serum could thus play a role in cancer detection as part of a panel of complementary serological markers that included assays for *p53* mutation, *K-RAS* mutation, and methylated *p16*. Moreover, our apparent current sensitivity for detecting later stage MSI colon cancers suggests that as currently constituted, serological assay of *hMLH1* promoter methylation could have potential application for the early

detection of postsurgical relapse of *hMLH1* methylated cancers. Additionally, our observations suggest the hypothesis that serological detection of *hMLH1* promoter methylation may also be of potential use as a prognostic marker for discriminating Dukes' C patients at high risk for distant metastases, who could benefit from postoperative chemotherapy, versus Dukes' C patients at lower risk for disseminated disease. Lastly, we cannot formally exclude that the methylated *hMLH1* DNA detected in serum might in some individuals reflect a constitutional susceptibility for aberrant methylation of this locus that extends to both gastrointestinal and lymphoid cells. Such questions will provide fruitful material for future studies.

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