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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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 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).

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 320ES 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 then performed 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
methylation of the hMLH1 gene

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 HpaII and MspI 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 HpaII (New England Biolabs), or 150 units of MspI (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 MgCl2, 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 2749A (5'-CCCTCATTATTCTGTCG) and 27266 (5'-TCAGTCGCCTGCTGCTC) 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 27589 (5'-TTCTTGGAGGCAGCACG) and 27860 (5'-CAATCATCCTAAATGCG), which amplify a 567-bp piece of yMSH2 that contains a HpaII 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 NH2 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, HpaII 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 is 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 HpaII and sensitive to digestion by MspI. In all cases, the promoter region of the control cell lines was sensitive to digestion by HpaII and MspI. In all experiments, the unmethylated internal control DNA was sensitive to

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<th>Transcription factor binding site consensus sequences present in the 5' region of hMLH1</th>
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<td>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.</td>
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digestion by HpaII and MspI. These data are consistent with methylation at CpG sites at all four HpaII 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 HpaII and sensitive to digestion by MspI, whereas DNA isolated from the normal tissue was sensitive to digestion by both HpaII and MspI. 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 HpaII and MspI. In the other case (Fig. 3, case 2), the DNA isolated from both the tumor and normal tissue was sensitive to digestion by both HpaII and MspI, although a small amount of HpaII-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 HpaII 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
endonucleases. Cases 1, 4, and 5 did not express hMLH1, and cases 2 and 3 digested with HpaII; M, digested with MspI.

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 of this goal of which is to determine the proportion and spectrum of sporadic cancers 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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