Altered Expression of hMSH2 and hMLH1 in Tumors with Microsatellite Instability and Genetic Alterations in Mismatch Repair Genes


Department of Laboratory Medicine and Pathology (S. N. T., A. J. F., P. C. R., J. M. C., D. J. T., L. J. B., R. H., K. C. H.) and Department of Medical Genetics (N. M. U, Mayo Clinic and Mayo Foundation, Rochester, Minnesota 55905; University of Dusseldorf, Dusseldorf, Germany [G. M.]; and Department of Molecular and Medical Genetics, Oregon Health Science University, Portland, Oregon 97207 [S. M. B., R. M. L.]

Abstract

To date, at least four genes involved in DNA mismatch repair (MMR) have been demonstrated to be altered in the germline of patients with hereditary nonpolyposis colon cancer: hMSH2, hMLH1, hPMS1, and hPMS2. Additionally, loss of MMR function has been demonstrated to lead to the phenomenon of microsatellite instability (MIN) in tumors from these patients. In this study, we have examined the protein expression pattern of hMSH2 and hMLH1 by immunohistochemistry in paraffin-embedded tumors from 7 patients with MIN+ sporadic cancer, 13 patients with familial colorectal cancer, and 12 patients meeting the strict Amsterdam criteria for hereditary nonpolyposis colon cancer. The relationship between the expression of these two gene products, the presence of germline or somatic mutations, and the presence of tumor MIN was examined. Nineteen of the 28 tumors studied demonstrated MIN, whereas mutations in hMLH1 and hMSH2 were detected in 6 and 2 patients, respectively. Of the eight MIN+/mutation+ cases, the absence of protein expression was observed for the corresponding gene product in all but one case (missense mutation in hMLH1). However, seven MIN+/mutation− cases also showed no expression of either hMLH1 (n = 5), hMSH2 (n = 1), or both (n = 1), whereas four MIN+ +mutation− cases demonstrated normal expression for both. None of the MIN−/mutation− cases (n = 9) demonstrated an altered expression pattern for either protein. These data suggest that examination of protein expression by immunohistochemistry may be a rapid method for prescreening tumors for mutations in the MMR genes.

Introduction

HNPCC is a clinically defined autosomal dominant disorder that accounts for approximately 6% of all cases of colon cancer. It is characterized by early age of onset, syn- and metachronous colorectal cancer, and cancers of other sites, including endometrium, stomach, small intestine, hepatobiliary tract, kidney, ureter, and ovary. A pedigree analysis is key in establishing the diagnosis of HNPCC. For comparative studies, the International Collaborative Group defined the Amsterdam criteria for the identification of HNPCC kindreds, namely: (a) at least three relatives should have histologically verified colorectal cancer, with at least two of them being first-degree relatives; (b) at least two successive generations should be affected; and (c) in one of the relatives, colorectal cancer should be diagnosed at under 50 years of age. Although the clinical definition of this syndrome was greatly facilitated by the establishment of the Amsterdam criteria, there remains a great deal of phenotypic variability among families. Furthermore, pedigree analysis is an imprecise tool, and this imprecision impairs the reliability of studies that address issues in HNPCC such as gene penetrance and tumor spectrum.

Recently, significant progress has been made in elucidating the underlying molecular basis of HNPCC (3–5). This genetic disorder can now be explained, in part, by the presence of germline mutations in one of at least four genes that participate in DNA mismatch repair, including hMSH2, hMLH1, hPMS1, and hPMS2 (6–9). Mutations in hMSH2 and hMLH1 are thought to account for approximately 60% of HNPCC kindreds, whereas only a few patients have had mutations in hPMS1 and hPMS2 (10). Interestingly, tumors from patients with HNPCC (11) and in a subgroup of patients with sporadic colon cancer (12, 13) have demonstrated a novel type of genetic instability characterized by alterations within microsatellites. Over the past three years, this phenomenon of MIN has been reported in an ever increasing number of tumor systems (3, 4, 14). Although defective mismatch repair is thought to be responsible for the MIN+ phenotype in tumors from patients with HNPCC, the genetic defect responsible for the MIN+ phenotype in sporadic colon cancer has yet to be clearly delineated.

The cloning and identification of DNA mismatch repair genes has facilitated identification of both germline and somatic mutations in individuals with HNPCC. However, the wide spectrum of mutations and the genetic heterogeneity involved with the disorder has been problematic. Furthermore, the methods available for the identification of specific gene mutations, such as DNA sequence analysis and coupled transcription-translation based assays, remain labor intensive. The presence of tumor MIN has offered a potential marker for the identification of individuals who are at high risk for possessing germline mutations in DNA mismatch repair genes. However, the presence of tumor MIN does not necessarily define which one of the four mismatch repair genes is involved, and more recent studies have not detected germline or somatic mutations in a substantial number of MIN+ sporadic colon cancer and MIN+ familial colorectal cancer (10, 15–18). An alternate method for the identification of defective mismatch repair would be to directly examine tumor specimens for the presence or absence of one of the DNA mismatch repair proteins itself. Additionally, this would allow for a more direct assessment of the specific gene involved. In this study, we examine the protein expression of hMLH1 and hMSH2 in paraffin-embedded tumors from patients with MIN+ sporadic colon cancer, patients with familial colorectal cancer, and patients meeting the strict Amsterdam criteria for HNPCC. The relationship between the expression of these two
EXPRESSION OF MISMATCH REPAIR GENES

Materials and Methods

Patient Population. Thirty-two patients were identified for this study. Twelve of these fulfilled the strict Amsterdam criteria (2) for HNPCC, 13 had a family history of colon cancer, and 7 had sporadic colon cancer. Familial cases were defined as those individuals having at least one other family member with colon cancer; in 9 of the 13 cases, this was a first-degree relative. For these familial cases, the average number of affected (all cancers) individuals per kindred was 6, with a range of 2–16 (including the proband), whereas the average number of colorectal cancers was 3, with a range of 2–7 (including the proband). When available, medical records were obtained on other affected family members to verify the presence of cancer. In most instances, however, such records were not available. Tissue used for these studies included fresh frozen tumor, paraffin-embedded tumor, and peripheral blood leukocytes.

Immunohistochemical Analysis. Immunoperoxidase staining for hMLH1 in formalin-fixed, paraffin-embedded tissue sections was performed by a labeled streptavidin-biotin method. Six-μm sections were mounted on silanized slides, deparaffinized, and rehydrated through graded alcohols to water. Endogenous peroxidase activity was blocked by incubation with 0.6% H2O2. Sections were immersed in 10 mM sodium citrate buffer, pH 6.0, and subjected to heat-induced antigen retrieval. Sections were then treated with 10% normal goat serum for 10 min to block nonspecific protein binding. Mouse monoclonal antibodies to hMLH1 (clone G168-728, 1 μg/ml, PharMingen) were applied, and tissue sections were incubated overnight at 4°C. After a brief rinsing, sections were treated with biotinylated antimouse IgG for 30 min at room temperature, rinsed, and then incubated with peroxidase-labeled streptavidin for 30 min at room temperature. After a brief washing, sections were incubated with diaminobenzidine and H2O2 for 5 min. Section were then lightly counterstained with hematoxylin, dehydrated in graded alcohols, cleared in xylene, and coverslipped. For immunoperoxidase staining of hMSH2, 6-μm sections were deparaffinized, rehydrated, blocked, and subjected to antigen retrieval as described above. Mouse monoclonal antibodies (Clone FE11, 0.5 μg/ml, Oncogene Science) were applied and tissue sections were incubated for 20 min at room temperature. After a brief rinsing, the Catalysed Signal Amplification system (DAKO Corp.) was used according to the manufacturer’s instructions for visualization of specific hMSH2 staining. Sections were counterstained and mounted as described for hMLH1. The normal staining pattern for both hMLH1 and hMSH2 was nuclear. Tumor cells that exhibited an absence of nuclear staining in the presence of nonneoplastic cells with nuclear staining were considered to have an abnormal pattern. Clone FE11 is a mouse monoclonal antibody generated with a carboxyl-terminal fragment of the hMSH2 protein, whereas clone G168-728 was prepared with full-length hMLH1 protein.

DNA Extraction. DNA extraction from fresh frozen tissue was performed as previously described (19). Normal and tumor DNA were also obtained from paraffin-embedded tissue. Using Tolu dine Blue-stained 10-μm-thick sections, areas of either normal or malignant tissue (≥70% neoplastic cells) were marked on the underside of the slide, and the tissue was scraped into a 1.5-ml microcentrifuge tube. Digestion was carried out by the addition of 20 mg/ml proteinase K in a 50 mM Tris buffer, pH 8.3 (2 μl proteinase K/100 μl buffer/1 cm² tissue) for 48 h at 55°C. Undigested tissue was removed by centrifugation, and proteinase K was inactivated by boiling for 8 min. Samples were rapidly cooled, and the DNA was stored at 4°C.

DNA Analysis. DNA sequence analysis and analysis for tumor MIN has previously been reported for this patient population (15). DNA sequence analysis was performed by the Sanger dyeoxy chain termination method with the use of the fmol sequencing kit (Promega, Madison, WI). For MIN, paired normal and tumor DNA were analyzed with a common set of 34 microsatellite markers. The number used ranged from a minimum of 9 to a maximum of 34 markers. All specimens were examined initially with the same group of 9 markers. MIN was defined by the presence of novel fragments following PCR amplification of tumor DNA that were not present in the PCR product generated by normal DNA. Tumors were considered MIN+ if 30% or more of the loci examined demonstrated instability.

<table>
<thead>
<tr>
<th>Case</th>
<th>Tissue</th>
<th>Gene</th>
<th>Exon</th>
<th>Codon</th>
<th>Base Changes</th>
<th>Consequence</th>
<th>MIN status</th>
<th>hMLH1</th>
<th>hMSH2</th>
</tr>
</thead>
<tbody>
<tr>
<td>2‖</td>
<td>T/N</td>
<td>hMLH1 (G)</td>
<td>13</td>
<td>499</td>
<td>Del G</td>
<td>Frameshift</td>
<td>Pos (7/8)</td>
<td>–</td>
<td>+</td>
</tr>
<tr>
<td>3&quot;</td>
<td>T/N</td>
<td>hMLH1 (G)</td>
<td>16</td>
<td>590-591</td>
<td>Del TAGA</td>
<td>Frameshift</td>
<td>Pos (7/8)</td>
<td>–</td>
<td>+</td>
</tr>
<tr>
<td>4&quot;</td>
<td>T/N</td>
<td>hMLH1 (G)</td>
<td>1</td>
<td>21</td>
<td>Del G</td>
<td>Frameshift</td>
<td>Pos (28/32)</td>
<td>–</td>
<td>NA</td>
</tr>
<tr>
<td>5&quot;</td>
<td>N</td>
<td>hMLH1 (G)</td>
<td>3</td>
<td>167</td>
<td>G → C</td>
<td>Missense, Asp → His</td>
<td>Pos (50)</td>
<td>–</td>
<td>+</td>
</tr>
<tr>
<td>6&quot;</td>
<td>N</td>
<td>hMLH1 (G)</td>
<td>8</td>
<td>226</td>
<td>C → T</td>
<td>Frameshift</td>
<td>Pos (50)</td>
<td>–</td>
<td>+</td>
</tr>
<tr>
<td>7&quot;</td>
<td>T/N</td>
<td>hMSH2 (So)</td>
<td>14</td>
<td>749</td>
<td>G → T</td>
<td>Frameshift</td>
<td>Pos (8/90)</td>
<td>–</td>
<td>+</td>
</tr>
<tr>
<td>8&quot;</td>
<td>N</td>
<td>hMLH1 (G)</td>
<td>5</td>
<td>492</td>
<td>A → T at 943 + 3</td>
<td>Frameshift</td>
<td>Pos (99)</td>
<td>–</td>
<td>+</td>
</tr>
</tbody>
</table>

* F, familial; S, sporadic; A, Amsterdam criteria; T, fresh frozen tumor; N, normal; So, somatic mutation; G, germline mutation; Del, deletion; Ins, insertion; Pos, positive; Neg, negative; NA, not available or no amplification.
* Number of markers showing instability/total number tested.
* Tissue available for sequence analysis.

Table 1. Sequencing, MIN, and clinical data

<ref>Downloaded from cancerres.aacrjournals.org on April 13, 2017. © 1996 American Association for Cancer Research.</ref>
Results and Discussion

In this study, we have evaluated the relationship between tumor MIN, the presence of germline or somatic mutations in hMSH2 and hMLH1, and the expression of the corresponding gene products in tumor tissues. Individuals were identified for this study based on either the presence of MIN in their tumor or the presence of a positive family history of colon cancer. Overall, there were 7 patients with sporadic colorectal cancer, 13 families with familial colon cancer, and 12 patients with HNPCC. Results of the DNA sequence analyses and analysis for tumor MIN in this group of patients (a subset of a larger group) have been reported previously (15).

Nineteen (68%) of the 28 tumors included in this study demonstrated widespread MIN (Table 1). Four additional tumors could not be evaluated due to amplification problems or the unavailability of appropriate tissue. Four of 9 tumors from patients with HNPCC, 8 of 13 tumors from patients with familial colon cancer, and all 7 tumors from patients with sporadic colon cancer were MIN+.

The hMLH1 and hMSH2 genes were sequenced in tumor and normal colonic DNA from 12 patients and in leukocyte DNA only from the other 20 (Table 1). Changes in the hMLH1 gene were detected in six patients (one sporadic, four familial, and one HNPCC), whereas two others showed a sequence change in hMSH2 (one familial, one HNPCC); two were somatic (case 1F and 2F), and six were germline alterations (Table 1). All but one of these mutations are predicted to result in a truncated protein, and thus, these are likely to be the causative mutations. The clinical significance of the one missense mutation (case 1S) is, at this point, unknown. It is of significance that all of the tumors with mutations in either hMSH2 or hMLH1 were MIN+.

Protein expression for hMLH1 and hMSH2 was examined in paraffin-embedded material from 32 and 28 patients, respectively (Fig. 1). In all sections examined, there were nonneoplastic cells that demonstrated positive nuclear staining for both hMSH2 and hMLH1. However, an absence of hMLH1 expression was observed in 10 tumors, an absence of hMSH2 expression in 3 tumors, and an absence of both in 1 tumor (Table 1). In all 14 cases, altered expression of either hMSH2 or hMLH1 was associated with tumor MIN. None of the MIN− tumors (n = 9) demonstrated an altered expression pattern for either protein. Of those MIN+ tumors having mutations in either hMLH1 (n = 6) or hMSH2 (n = 2), the absence of one of the two mismatch repair gene products was associated with the presence of a mutation in the corresponding gene in all but one case (Table 1). In this one discordant case (case 1S), the patient had a missense mutation in exon 13 of hMLH1 and normal hMLH1 expression in the tumor. In addition to the MIN+/mutation+ cases that showed no detectable protein expression with the neoplastic cells, seven MIN+/mutation− cases also showed no expression of either hMLH1 (n = 5), hMSH2 (n = 1), or both (n = 1), whereas four MIN+/mutation− cases demonstrated normal expression of both proteins.

These results confirm and extend those observations first made by Leach et al. (20), in which hMSH2 immunoreactivity was not detected in colorectal tumors from a few patients with germline hMSH2 mutations. Our study, however, extends these initial observations by examining the expression pattern of both hMSH2 and hMLH1 in a larger series of patients and correlating these changes with the presence of gene mutations and tumor MIN. Our results showed that an absence of protein expression for both hMSH2 and hMLH1 in neo-
plastic cells was associated with the presence of widespread tumor MIN. All 14 cases lacking protein expression had MIN+ tumors, whereas all of the tumors that were MIN− (n = 9) had normal protein expression patterns for both proteins. Additionally, the presence of a germline or somatic mutation in one of the DNA mismatch repair genes, hMSH2 and hMLH1, was highly associated with the presence of both tumor MIN and the absence of protein expression in the corresponding gene product. All eight cases with either a somatic or germline mutation had MIN+ tumors, and in all but one case, an absence of protein expression was observed. Because none of the MIN− cases had altered protein expression, the immunohistochemical analysis of tumor tissue may be a useful screen for the detection of defective mismatch repair. Additionally, this may be helpful for the identification of a high-risk group of individuals having HNPCC. Other than family history, there is still a lack of useful markers for establishing the diagnosis of HNPCC.

It is important to note that all but one of the hMSH2 and hMLH1 gene alterations identified in this study are predicted to result in a truncated gene product. The lack of protein expression in the tumor from these cases suggests that aberrant hMSH2 and hMLH1 polypeptides or the mRNAs encoding them are unstable (20). Although the clinical significance of the one missense mutation detected is unknown, such mutations may give rise to normal protein levels but abnormal function. The immunohistochemical detection of protein, therefore, does not necessarily imply normal DNA mismatch repair function. These cases, however, would be predicted to still maintain their MIN+ tumor phenotype. It is also interesting to note that individuals with germline mutations (i.e., heterozygous) in either hMSH2 or hMLH1 have normal expression in nonneoplastic colonic tissue and no expression in the adjacent adenocarcinoma. These data further support the idea that both alleles of the mismatch repair genes need to be eliminated for abnormal function.

Not all of the tumors that lacked expression of hMSH2 or hMLH1 protein had identifiable germline or somatic mutations. There were seven such cases: four sporadic, two familial, and one HNPCC. These individuals, however, may have mutations that were not detected by the sequencing method used, such as deletions of entire exons (10) or alterations in the promoter region. For three of the seven cases, tumor DNA was not available for study. The lack of protein expression in these cases, therefore, might be explained by the presence of somatic alterations in either hMSH2 or hMLH1. Alternatively, there may be other proteins or factors that are involved in the regulation or stabilization of hMSH2 or hMLH1 protein levels. Alterations of these components may influence both the amount of mismatch repair protein and subsequently MIN. It is interesting to note that of the seven MIN+ mutation cases that lacked protein expression, hMLH1 was involved in the majority (n = 6).

Four MIN+ mutation cases had normal expression of both gene products. In three of these, DNA from tumor was also available for sequence analysis, ruling out the presence of both germline and somatic alterations. Although it is possible that missense mutations in hMSH2 or hMLH1 might have been missed, these data more likely suggest that alterations in other mismatch repair genes, such as hPMS1, hPMS2, or genes as yet unidentified, may be involved in generating the MIN+ phenotype.

The availability of antibody for both hMSH2 and hMLH1 should facilitate the study of a large number of patients with familial colon cancer. Furthermore, it will be important to further study such cases with antibody directed against hPMS1 and hPMS2. To date, the presence of germline mutations in hPMS1 and hPMS2 has been identified in only a few patients (7, 10). The role of these two genes in HNPCC and MIN, therefore, remains to be clarified. The analysis of protein expression for both hPMS1 and hPMS2 by immunohistochemistry in MIN+ tumors and tumors from patients with familial colon cancer should help to resolve some of these issues.

Overall, one-half of the tumors with abnormal hMLH1 or hMSH2 protein expression had a mutation in the corresponding gene, and a mutation in either gene resulted in abnormal expression in all but one case. There was no case in which DNA sequence analysis implicated one gene and immunohistochemical analysis implicated the other. Because antibody staining is more available than DNA analysis in a clinical setting, the use of immunohistochemistry appears to offer a relatively convenient and rapid method for prescreening tumors for defects in the expression of mismatch repair genes. Ultimately, this technique, along with testing for tumor MIN, should help to identify those individuals who may have germline mutations in the mismatch repair gene complex. However, verification of these findings in a much larger number of patients is necessary. Furthermore, it will also be important to identify the underlying molecular defect in those MIN+ tumors that have an absence of protein expression but no apparent gene mutations.

References
EXPRESSION OF MISMATCH REPAIR GENES

16. Liu, B., Nicolaides, N. C., Markowitz, S., Willson, J. K. V., Parsons, R. E., Jen, J.,
    Papadopoulos, N., Peltonäki, P., de la Chapelle, A., Hamilton, S. R., Kinzler, K. W.,
    and Vogelstein, B. Mismatch repair gene defects in sporadic colorectal cancers with

    Kane, M. F., Rognum, R. O., and Kolodner, R. D. Somatic mutations in the hMSH2

    and Hirohashi, S. Clinical implications of microsatellite instability in colorectal

19. Cunningham, J., Lust, J. A., Schaid, D. J., Bren, G. D., Carpenter, H. A., Rizza, E.,
    Kovach, J. S., and Thibodeau, S. N. Expression of p53 and 17p allelic loss in

    A. H., Peltonäki, P., de la Chapelle, A., Hamilton, S. R., Kinzler, K. W., and
    Vogelstein, B. Expression of the human mismatch repair gene hMSH2 in normal and
Altered Expression of hMSH2 and hMLH1 in Tumors with Microsatellite Instability and Genetic Alterations in Mismatch Repair Genes

Stephen N. Thibodeau, Amy J. French, Patrick C. Roche, et al.

*Cancer Res* 1996;56:4836-4840.

**Updated version**
Access the most recent version of this article at:
http://cancerres.aacrjournals.org/content/56/21/4836

**E-mail alerts**
Sign up to receive free email-alerts related to this article or journal.

**Reprints and Subscriptions**
To order reprints of this article or to subscribe to the journal, contact the AACR Publications Department at pubs@aacr.org.

**Permissions**
To request permission to re-use all or part of this article, contact the AACR Publications Department at permissions@aacr.org.