Defective Mismatch-Repair Colorectal Cancer

Clinicopathologic Characteristics and Usefulness of Immunohistochemical Analysis for Diagnosis

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Abstract

The purpose of our study was to determine the usefulness of immunohistochemical analysis for the diagnosis of mismatch-repair (MMR) gene defective colorectal tumors and to describe their prevalence and clinicopathologic characteristics. We studied 172 cases. DNA was extracted from formalin-fixed, paraffin-embedded surgical samples, and microsatellite analysis was performed by polymerase chain reaction with BAT-26. The results were correlated with immunohistochemical analysis for hMLH1 and hMSH2. Microsatellite instability (MSI) was detected in 13 (7.6%) tumors, and all showed loss of protein expression of hMLH1 (11/13) or hMSH2 (2/13) (P < .000). Patients with MMR-defective tumors more frequently had poorly differentiated tumors (5/13 [38%] vs 18/159 [11.3%; P = .02) located in the ascending colon (8/13 [62%] vs 30/159 [18.9%; P < .0001) and a personal history of other neoplasms (4/13 [31%] vs 18/159 [11.3%; P = .05). There were no differences in age, family history of cancer, or TNM stage. Immunohistochemical analysis seems to be a reliable method to detect most colorectal cancers with defective MMR genes.

Colorectal cancer (CRC) is the third most common type of cancer in Western Europe and an important cause of death in our society. It is known that there are at least 2 completely different pathogenetic pathways for CRC. The most common genetic alterations, occurring in approximately 85% of the cases, are allelic losses (so-called loss of heterozygosity), chromosomal amplifications and translocations, that affect mainly tumor-suppressor genes (APC, p53, SMAD4) and some oncogenes (k-ras).1,2 These alterations are characteristic of the chromosomal instability pathway.

The remaining 15% display frame-shift mutations and base-pair substitutions in microsatellites,3,4 which are repetitive genetic loci with 1 to 5 base pairs repeated 15 to 30 times. Microsatellites are prone to slippage during DNA replication, resulting in small loops in the DNA strand. These errors are normally controlled and repaired by the DNA mismatch-repair (MMR) genes such as hMLH1, hMSH2, hMSH6, hPMS2, and hMSH3.4 This form of genetic destabilization is caused mainly by their loss of function, and it is referred to as the microsatellite instability (MSI) pathway. MSI is present in virtually all the cases of hereditary nonpolyposis colorectal cancer (HNPCC) and approximately 15% of sporadic cases. Almost all CRC cases display chromosomal or microsatellite instability, and an inverse relationship exists between these 2 pathways of carcinogenesis. MSI cancers are diploid and show normal rates of gross chromosome aberrations, whereas chromosomal unstable cancers are aneuploid and show increased rates of chromosomal losses and gains.1

The 3 ways to study alterations of the MMR mechanisms are (1) sequencing of the genes involved, (2) detection of the gene products or proteins, and (3) analysis of the microsatellites. The most common method to study tumors with DNA
MMR impairment is molecular analysis. The National Cancer Institute published criteria for the determination of MSI in CRC. After testing with a panel of 5 microsatellites (BAT-25, BAT-26, D2S123, D5S346, and D17S250), a tumor is described as having MSI if 2 or more of the 5 markers are unstable. Several studies have used only BAT-26 because it is considered the most sensitive and specific marker, is able to identify more than 95% of MSI cases, and it does not require nontumoral DNA for comparison.

Testing for MSI would serve at least 2 purposes. First, it is a powerful method to screen for HNPCC because evidence suggests that members of families with HNPCC benefit from clinical screening by colonoscopy. Second, MSI sporadic tumors have a better prognosis and an uncertain response to chemotherapy. A recent study suggested that fluorouracil-containing chemotherapy is associated with a better prognosis and an uncertain response to chemotherapy.11,12 A recent study suggested that fluorouracil-based chemotherapy does not increase survival of patients with MSI-defective CRC.13 Therefore, the knowledge of MSI status could help physicians assess prognosis and, perhaps, could guide their therapy.14 Testing for MSI is time consuming, expensive, and not available in most routine diagnostic laboratories. Development of novel antibodies against some MSI genes, such as hMLH1 and hMSH2, might permit the detection of underlying alterations in the genes by immunohistochemical analysis.

The aims of our study were to determine the usefulness of immunohistochemical analysis for the diagnosis of defective MSI genes in a series of CRC with known MSI status and to describe their prevalence and clinicopathologic characteristics. Our results showed excellent correlation between MSI status and hMLH1 and hMSH2 protein expression, supporting immunohistochemical analysis as a rapid, easy, and reliable method to detect CRC defective in MSI genes.

**Materials and Methods**

**Cases and Tumors Samples**

We included 172 cases of CRC. All patients were diagnosed and treated surgically from October 1999 to June 2002. Patients were included consecutively in 2 periods: October 1999 to February 2000 (37 patients) and October 2000 to June 2002 (135 patients). Clinical records were reviewed, and data regarding familial and personal history of cancer were recorded. Tumor-related parameters included location, size, TNM stage (American Joint Committee on Cancer), grade of differentiation, lymphocytic infiltration, and the presence of signet-ring cells. Pedigrees were traced backward and laterally as far as possible or at least up to second-degree relatives with respect to oncologic history. Age at cancer diagnosis, tumor location, histologic type, and stage of the neoplasm were recorded for each affected family member. The definition of HNPCC was based on Amsterdam II criteria.16 HNPCC-related neoplasms included CRC and cancer of the endometrium, small bowel, ureter and renal pelvis, ovary, stomach, and hepatobiliary system, according to Bethesda guidelines.17

**DNA Extraction, Microsatellite Markers, and MSI Analysis**

DNA was extracted from paraffin-embedded tissue samples according to a previously described method based on microwave irradiation without deparaffinization and incubation with Proteinase K at 48°C overnight.18 MSI status was determined by using the microsatellite marker BAT-26. We used this method based on previous articles that reported a high sensitivity and specificity for detecting MSI using only BAT-26.19,20 This microsatellite marker is quasi-monomorphic and permits performance of the MSI test using only tumor DNA, without the requirement for matching normal DNA.8,10,21 Thermocycling conditions were 94°C for 5 minutes, followed by 35 cycles of 94°C, 55°C, and 72°C for 20 seconds each, and final incubation for 5 minutes at 72°C. Polymerase chain reaction products were loaded on 10% polyacrylamide sequencing gels and visualized by silver staining. Tumors exhibiting MSI showed polymerase chain reaction products shorter than those of normal tissue control samples.

**Immunohistochemical Analysis**

One block of formalin-fixed, paraffin-embedded tumor tissue was selected per case. We dewaxed and rehydrated 4-µm-thick sections using xylene and alcohol. Before immunostaining, antigen retrieval was performed by immersing sections in a 10-mmol/L concentration of citrate buffer, pH 6.0, and boiling in a pressure cooker for 5 minutes. Sections then were incubated for 20 minutes at room temperature with mouse monoclonal antibodies against hMLH1 protein (clone G168-15, dilution 1:40; PharMingen, San Diego, CA) and hMSH2 protein (clone FE11, dilution 1:35; Oncogene Research Products, Boston, MA). An UltraVision streptavidin-biotin peroxidase detection kit (Dako, Carpinteria, CA) was used as the secondary detection system. The peroxidase reaction was developed using diaminobenzidine tetrachloride as the chromogen. Loss of hMLH1 or hMSH2 expression was recorded when there was a complete absence of nuclear staining in neoplastic cells.

**Image 11.** Nuclear staining in normal epithelial cells, lymphocytes, and stromal cells in each slide served as internal positive controls.

**Statistical Analysis**

Descriptive statistics were calculated to characterize the entire data set. Comparison between variables was done using the $\chi^2$ test for qualitative variables and the Student $t$
test or Mann-Whitney U test for quantitative variables. Relative risk (RR) and 95% confidence interval (CI) were also calculated. MSI results were used as the “gold standard” to define the sensitivity and specificity of immunohistochemical analysis. All results were calculated using SPSS 10.0 statistical software (SPSS, Chicago, IL). *P* values of less than .05 were considered statistically significant.

### Results

In our series of 172 CRC cases, the mean ± SD age of the patients was 67.8 ± 11.8 years; the TNM stage was I in 2.3% of the cases, II in 47.1%, III in 29.1%, and IV in 21.5%. Of the 172 tumors, 51 (29.7%) were proximal to the splenic flexure, and 121 (70.3%) were distal. The median ± SD tumor size was 51 ± 21 mm. Most tumors (146, 84.9%) were classified as conventional adenocarcinoma, 23 (13.4%) were of the mucinous type, and 3 (1.7%) were medullary carcinoma. The Amsterdam II criteria were fulfilled by 3 cases (1.7%), and 21 (12.2%) fulfilled one or more Bethesda guidelines.

Twenty-two patients (12.8%) had a history of cancer. Tumor locations were as follows: colorectal, 10; endometrial, 3; breast, 6; lung, 2; and kidney, bladder, and vocal cord, 1 case each. One patient had 3 previous tumors (colon, endometrium, and breast).

Sixty-three patients (36.6%) had a first-degree relative with a history of cancer, and 24 (14.0%) had more than one first-degree relative with a history of cancer. Twenty-nine patients (16.9%) had at least 1 first-degree relative with a history of cancer.

![Image 1](Image 1)

**Image 1** Immunostaining for hMLH1 and hMSH2. A (Case 1), Loss of expression of hMLH1. B (Case 1), Normal expression of hMSH2. C (Case 12), Normal expression of hMLH1. D (Case 12), Loss of expression of hMSH2.
HNPCC-related cancers (CRC, 20; endometrial carcinoma, 9; gastric carcinoma, 6; ovarian, 2; urothelial tumor, 1). Among those patients, 8 had more than one first-degree relative with HNPCC-related cancers.

We found MSI in 13 tumors (7.6%), and all showed loss of expression of hMLH1 (11/13 [85%]) or hMSH2 (2/13 [15%]). No CRC that showed normal expression for hMLH1 and hMSH2 exhibited MSI. Correlation between methods was 100% (P < .000). Clinical and pathologic characteristics of the patients according to MMR gene and MSI status are given in Table 1 and Table 2. Of 8 patients with more than one first-degree relative with HNPCC-related cancer, 3 had tumors with MSI and fulfilled the Amsterdam II criteria. One patient without a family history of neoplasms had a loss of expression of hMSH2 (Table 2, case 12). MMR-defective tumors more often were located in the ascending colon (P < .05; RR, 3.26; 95% CI, 1.91-5.58) and were high-grade (P < .05; RR, 3.39; 95% CI, 1.50-7.66) (Table 1). In addition, patients with MMR-defective tumors more frequently had a history of cancer (P < .05; RR, 2.72; 95% CI, 1.08-6.85) (Table 1).

Of note, the 3 cases of medullary-type carcinoma detected in our series showed loss of expression of hMLH1. We found no differences in age, sex, tumor size, TNM stage, mucin production, or history of cancer in first-degree relatives when the results were compared with those of patients with non–MMR-defective tumors (all P > .05).

**Discussion**

Several studies have reported that defects in MMR genes are the basis of MSI, which is present in virtually all cases of HNPCC and a subset of sporadic CRC cases.22,23 Based on our molecular and immunohistochemical analyses of 172 CRC cases, it seems that in a small proportion of tumors, alteration of the MMR genes (hMLH1 and to a lesser extent hMSH2) in association with MSI occurs. MSI was found in 7.6% of the cases, which is lower than the rates reported by other authors in nonselected populations (12%-20%).7,9,22,23 We performed the analysis of MSI with the mononucleotide marker BAT-26 because of its reported high sensitivity and specificity, which ranges between 96% and 100%8,10,19-21,24; for this reason, the use of BAT-26 is considered sufficient for screening for CRC for mismatch-repair deficiency.21 However, our frequency of MSI is lower than found in previous studies in nonselected populations, also done with only BAT-26 and showing MSI in 12% to 15% of CRC cases.7,9

Interestingly, studies done in the Mediterranean area have shown lower rates of MSI than others done in North America.

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**Table 1**

<table>
<thead>
<tr>
<th>Characteristics of Patients and Tumors Based on MSI/MMR Gene Defects*</th>
</tr>
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<tbody>
<tr>
<td><strong>Non–MMR-Defective</strong></td>
</tr>
<tr>
<td><strong>Tumors (n = 159)</strong></td>
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<tr>
<td>Mean ± SD age (y)</td>
</tr>
<tr>
<td>Sex (M/F)</td>
</tr>
<tr>
<td>Location</td>
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<tr>
<td>Ascending</td>
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<tr>
<td>Transverse</td>
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<tr>
<td>Descending</td>
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<tr>
<td>Rectum</td>
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<tr>
<td>High-grade</td>
</tr>
<tr>
<td>Mucinous tumors</td>
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<tr>
<td>Stage (I/II/III/IV)</td>
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<tr>
<td>I</td>
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<td>II</td>
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<tr>
<td>III</td>
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<tr>
<td>IV</td>
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<tr>
<td>Size (mm)</td>
</tr>
<tr>
<td>Amsterdam criteria met</td>
</tr>
<tr>
<td>Bethesda criteria met</td>
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<tr>
<td>Personal history of neoplasia</td>
</tr>
<tr>
<td>Family history of neoplasia</td>
</tr>
<tr>
<td>Family history of HNPCC-related tumors</td>
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</tbody>
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* Numbers in parentheses are percentages.
* P < .05 for differences.

**Table 2**

<table>
<thead>
<tr>
<th>Characteristics of Patients With MMR-Defective Tumors</th>
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<tbody>
<tr>
<td><strong>Case No./Sex/Age (y)</strong></td>
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<tr>
<td>----------------------------</td>
</tr>
<tr>
<td>1/M/50</td>
</tr>
<tr>
<td>2/F/78</td>
</tr>
<tr>
<td>3/F/77</td>
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<tr>
<td>11/F/86</td>
</tr>
<tr>
<td>12/F/73</td>
</tr>
<tr>
<td>13/F/88</td>
</tr>
</tbody>
</table>

A, ascending colon; D, descending colon; MMR, mismatch repair; T, transverse colon; +, positive; –, negative.
America or northern Europe. For example, an Italian study reported MSI in 8.3% of the tumors, similar to our results. It is thought that dietetic, toxic, or other environmental factors can cause epigenetic disruption of hMLH1, such as promoter hypermethylation of the gene, influencing in this manner the proportion of MMR-defective tumors. This could explain the lower rates of MSI cases in the Mediterranean countries, where some dietary habits that could be related to MSI are less frequent. For example, red meat ingestion, a high frequency of using cooking practices that increase the intake of heterocyclic amines (eg, frying, barbecuing, or broiling), and consumption of high-grade alcoholic drinks are found in countries where MSI rates are higher.

Although the diagnosis of CRC at a younger age is frequent in HNPCC, sporadic MMR-defective tumors can be diagnosed at any age. In our study, the mean age at diagnosis was high (70.5 years), even in patients who fulfilled the Amsterdam criteria (Table 2). This fact has clinical importance, because advanced age at diagnosis of CRC does not exclude completely the possibility of HNPCC.

MMR status analysis in CRC has become an important issue because the response to chemotherapy and the prognosis for patients with and without MSI are different. The gold standard for diagnosis of MMR-defective tumors is still molecular testing. We found an excellent correlation between MSI status detected with BAT-26 and hMLH1 and hMSH2 protein expression, and that finding confirms the reliability of immunohistochemical analysis. Our results are in line with those of previous studies that have shown good sensitivity and specificity ranging between 90% and 100%. Debnik et al estimated that immunohistochemical analysis costs only 14% to 28% of what MSI testing costs. In addition, this method is far less labor intensive, and it is available to any pathology laboratory. Additional advantages are its usefulness for the study of MMR status in cases with scarce tumor tissue, such as mucinous carcinomas and radiation-treated tumors, and specific detection of the impaired protein, which is important to determine strategies for germline testing. For example, the loss of expression of hMSH2 frequently is associated with germline mutations. Therefore, it is possible to suspect HNPCC by immunohistochemical analysis only and to recommend screening family members of patients suspected of having HNPCC. In fact, we observed absence of hMSH2 in 1 tumor (Table 2, case 12); however, our patient had no family history of neoplasm and did not meet Bethesda criteria.

MMR-defective sporadic and hereditary tumors have special pathologic characteristics. They frequently are located in the ascending colon, are large and high-grade, and have signet-ring cells and prominent lymphocytic intratumoral and peritumoral infiltration (Crohn-like). The presence of undifferentiated or medullar carcinoma with a trabecular growth pattern and significant intratumoral lymphocytic infiltrate have been associated with MSI phenotype. This histologic type frequently shows loss of hMLH1 expression in nonselected patients, but in those with HNPCC, it frequently is associated with germline mutation in hMSH2.

In our series, we found 3 cases of medullary carcinoma, all with loss of expression of hMLH1, and none fulfilled the Amsterdam criteria for HNPCC. On the other hand, mucinous carcinomas are known to be associated with defects in the MMR system. Our results are in disagreement, because only 1 of 23 mucinous carcinomas showed MSI and loss of expression of hMLH1. It is possible that in this histologic type the relatively scarce tumor cells could be lost during molecular analysis, resulting in false-negative MSI results. However, immunohistochemical analysis confirmed the normal expression of the proteins in tumor cells.

Immunohistochemical analysis is a useful and easy method that can be performed routinely in all sporadic CRC cases for the detection of MMR-defective tumors. Moreover, it might provide relevant information about underlying germline mutations, response to treatment, and prognosis. In cases that have normal expression of hMLH1 or hMSH2 but possibly are HNPCC or sporadic MMR-defective tumors, further molecular analysis should be performed.

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Jover et al. / IMMUNOHISTOCHEMICAL ANALYSIS IN MMR-DEFECTIVE COLORECTAL CANCER


