Diagnostic Microsatellite Instability: Definition and Correlation with Mismatch Repair Protein Expression

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ABSTRACT

Alterations of the length of simple repetitive genomic sequences (microsatellite instability, MSI) characterize a distinct mechanism of colorectal carcinogenesis. Such MSI has been found to be associated with hereditary nonpolyposis colorectal cancer (HNPCC) which involves mutation of the human mismatch repair genes hMSH2 and hMLH1 as well as many sporadic cancers of most tissue types. Although the study of MSI diagnosis is a useful tool for HNPCC screening and for the determination of tumor prognosis in sporadic cases of colorectal cancer, the reliability of MSI diagnosis is still a subject of debate. Here we have examined 58 primary colorectal tumors (selected from a cohort of 200) using 31 microsatellite markers that comprised the most frequent simple repeat types. The expression of the hMSH2 and hMLH1 mismatch repair proteins was studied by immunohistochemistry, and most patients were surveyed for at least 2 years. Reproducibility of gel interpretation, as well as diagnostic sensitivity and specificity of the MSI status, were determined. We found that unambiguous determination of band shifts as well as MSI diagnosis were closely related to the type of the marker repeat and that MSI could be subdivided into “high” MSI (>20% unstable loci), “low” MSI (<10% unstable loci), and microsatellite stable (0% unstable loci). One-half of the patients with high MSI tumors (n = 8) fulfilled either the Amsterdam criteria (n = 4), had at least one relative with HNPCC-related carcinoma (n = 2), or were diagnosed with colorectal cancer at an age below 45 years (n = 2). Fifteen of the 15 high MSI tumors had lost either hMSH2 (n = 8) or hMLH1 (n = 6) protein expression. In contrast, all of the low MSI tumors and the MSI-negative tumors displayed normal expression of hMSH2 and hMLH1. These studies provide a clear recommendation for the uniform use of a panel of 10 microsatellites and a definition of at least 40% instability (using these defined marker loci) in the diagnostic analysis of MSI.

INTRODUCTION

MSI1 is characterized by small deletions or expansions within short tandem repeats in tumor DNA as compared with matching normal DNA. Tumors with MSI have been classified as “microsatellite unstable” (MIN) or “replication error positive” (RER+) (1, 2). The use of these two nomenclatures has appeared confusing because microsatellite instability, MSI) characterize a distinctive mechanism of colorectal carcinogenesis. Such MSI has been found to be associated with hereditary nonpolyposis colorectal cancer (HNPCC) which involves mutation of the human mismatch repair genes hMSH2 and hMLH1 as well as many sporadic cancers of most tissue types. Although the study of MSI diagnosis is still a subject of debate. Here we have examined 58 primary colorectal tumors (selected from a cohort of 200) using 31 microsatellite markers that comprised the most frequent simple repeat types. The expression of the hMSH2 and hMLH1 mismatch repair proteins was studied by immunohistochemistry, and most patients were surveyed for at least 2 years. Reproducibility of gel interpretation, as well as diagnostic sensitivity and specificity of the MSI status, were determined. We found that unambiguous determination of band shifts as well as MSI diagnosis were closely related to the type of the marker repeat and that MSI could be subdivided into “high” MSI (>20% unstable loci), “low” MSI (<10% unstable loci), and microsatellite stable (0% unstable loci). One-half of the patients with high MSI tumors (n = 8) fulfilled either the Amsterdam criteria (n = 4), had at least one relative with HNPCC-related carcinoma (n = 2), or were diagnosed with colorectal cancer at an age below 45 years (n = 2). Fifteen of the 15 high MSI tumors had lost either hMSH2 (n = 8) or hMLH1 (n = 6) protein expression. In contrast, all of the low MSI tumors and the MSI-negative tumors displayed normal expression of hMSH2 and hMLH1. These studies provide a clear recommendation for the uniform use of a panel of 10 microsatellites and a definition of at least 40% instability (using these defined marker loci) in the diagnostic analysis of MSI.

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1 The abbreviations used are: MSI, microsatellite instability; APC, adenomatous polyposis coli; HNPCC, hereditary nonpolyposis colorectal cancer; MSS, microsatellite stable.

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19107 [T.B., R.F.]

MATERIALS AND METHODS

Tumors. Fifty-eight primary colorectal adenocarcinomas were selected from an ongoing prospective study of about 200 cases, which were initially tested for five randomly chosen dinucleotide markers (APC, D5S171, TP53, Mfd 26, and Mfd 28). Of these 200 cases, 24 (12%) displayed MSI at two or more loci, 25 (12.5%) displayed MSI at only one loci, and 151 (75.5%) were scored as MSS. The selection of the 58 colorectal cancer cases used for this study from the ongoing prospective study of 200 cases was based on obtaining an approximately equal distribution of MSI and MSS cases for further evaluation of the MSI technology. These colorectal tumors were initially scored as MSI if there were at least two unstable loci (n = 14), as indefinite for MSI if only one unstable locus (n = 9) was present, and as negative if no instabilities at the five tested microsatellite loci (n = 35) were found. A thorough family history was then obtained for all patients as well as follow-up information that included data from 14 to 51 months (median, 29 months) after presentation.

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Four patients fulfilled the Amsterdam criteria for HNPCC (22), five patients had a family history of at least one relative suffering from a HNPCC-related tumor (endometrium, gastric, or colorectal carcinoma), and one patient had three metachronous carcinomas (colorectal, endometrial, and bladder cancer) not meeting the strict Amsterdam criteria (Table 2, patient 38). Five patients were of young age lower than 45 years, and 43 had sporadic colon cancer without any of the aforementioned features.

Microsatellite Analysis. For MSI analysis, normal and tumor DNA were extracted from paraffin-embedded tissue as described previously (23). Matched normal and tumor DNA were investigated with a set of 31 microsatellite markers (Table 1). In contrast to the commonly used dinucleotide primers, the primer panel was chosen with respect to their repeat motif comprising five different repeat types: 6 loci with mononucleotide runs (BAT25, BAT26, BAT40, 52H10, 52C10, and D1) and 15 loci with CA dinucleotide repeats (APC, Mfd15, Mfd26, Mfd28, Mfd41, D2S123, D3S1283, D8S171, D10S197, D11S1318, D11S904, D13S175, D18S58, D18S69, and TP53). Three loci exhibited trinucleotide (AR, TRP, and SR), five loci tetranucleotide (HPRT1, HPRT2, MYCL1, RB, and TBP), and two loci pentanucleotide repeats (FMR2 and TP53). Each sequence of these loci was exactly examined by GenBank data or by sequencing PCR products if no sequence data were available. Interestingly, all but six dinucleotide loci (APC, D13S175, D3S1283, Mfd26, Mfd28, and Mfd41) showed additional nucleotide repeats of different range and type flanking the "main repeat." Thus, dinucleotide microsatellite loci were defined as complex (additional repeat structures present) and noncomplex ("pure") repeats.

PCR amplifications were performed with 100 ng of purified genomic DNA in a final volume of 20 µl in an MJ Research Thermocycler (PTC100; MJ Research, Watertown, MA). Subsequently, PCR products were analyzed by 6.7% polyacrylamide/50% urea gel electrophoresis (1 h, 1500 V, 50°C) in a SequiGen sequencing gel chamber (Bio-Rad, Hercules, CA) and by silver nitrate staining as described previously (24).

MSI was defined by the presence of novel bands following PCR amplification of tumor DNA, which were not present in PCR products of the corresponding normal DNA. To determine those markers in which band shifts are difficult to interpret, all gels were evaluated independently by three different observers (W. D., S. W., and J. R.). Every locus was scored as MSI, MSS, or uncertain in all 58 tumors. An "uncertainty score" was derived for every single marker by assessing the frequency of unequivocally detected MSI and those in which the MSI status was described as uncertain or different by at least one of the three observers. Finally, a tumor was considered MSI if more than 20% of the 31 examined loci showed unequivocal instabilities, whereas less than 20% instability was classified as uncertain; MSS was classified if no microsatellite loci were found to be unstable.

Immunostaining for hMLH1 and hMSH2. To determine the mismatch repair protein status, all tumors were subjected to immunohistochemical analysis using the streptavidin-biotin-peroxidase complex method with 3,3′-diaminobenzidine as chromogen. Four-µm-thick sections of 10% neutral buffered formalin-fixed, paraffin-embedded tumor tissue were incubated overnight at 4°C with a polyclonal rabbit antibody against the hMLH1 (clone G168-728; 1 µg/ml; PharMingen, San Diego, CA). After deparaffinization, the sections were pretreated by microwave (four times for 4 min at 900 W in 0.1 M citrate buffer). Normal mouse sera were used as negative controls. Infiltrating lymphocytes as well as normal colonic crypt epithelium next to the tumor served as internal positive controls.

RESULTS

Reliability of Band Shift Interpretation. Two different band patterns were found comprising those with distinct alleles and those with scattered bands (laddering) for all of the 31 microsatellite markers (Fig. 1). Although scattered bands tended to be more difficult in the interpretation, the repeat motif turned out to be the major determinant of MSI both with respect to the detection frequency as well as the reliability of interpretation. For example, noncomplex dinucleotides provided the highest uncertainty score (22 uncertain versus 39 certain band shifts; i.e., 56.5%). These markers were followed by complex dinucleotides with an intermediate uncertainty score (19 uncertain versus 74 certain instabilities; i.e., 25.6%). The corresponding values were 14.7% for all tri-, tetra-, and penta-repeat types and were lowest in the mononucleotide repeat markers (10.9%; Table 2). Loci with many clear shifts (≥10) and only few equivocal shifts (≤1) were seen in almost all mononucleotide repeats (BAT25, BAT26, BAT40, 52C10, and 52C10), in one complex dinucleotide repeat (D18S58) and in one tetranucleotide repeat (MYCL1). Most equivocal shifts with high interobserver variability were seen in D1, APC, D13S175, D11S904, HPRT II, D3S1283, Mfd28, and Mfd41, respectively.

MSI Detection Frequency per Microsatellite Primer. Although only unequivocally detected MSI were considered, the number of instabilities per locus varied substantially, ranging from no shifts in HPRT1, HPRT2, FMR2, SR, and LEN, to 14 shifts with the APC primer. Thus, a close relationship between the type of repeat motif and the number of detected MSIs was evident. The mean detection frequency per marker was highest in mononucleotide repeats (10.6 per marker) and lowest in tetra- and pentanucleotide repeats (2.6 and 3.0 per marker, respectively).

With regard to the 15 dinucleotide markers, the complex dinucleotide microsatellite loci appeared to produce more instabilities than noncomplex markers (6.5 versus 8.2). The mononucleotide repeat sequences amplified by BAT26 and BAT40 showed the clearest shifts, where the extent of band shifting increased with the length of the mononucleotide run. Interestingly, all shifts displayed deletions and no allele enlargement. In loci with less than 10 repetitive mononucleotides (e.g., hMSH3, hMSH6, and BAX), only very small shifts occurred, which were difficult to be detected in standard gels (data not shown). The significance of mononucleotide runs in MSI analysis was further substantiated when other complex repeat loci were compared for instability frequency. Although detection frequency in tetra- and pentanucleotide repeats was quite low, one marker, MYCL1, showed a relatively high instability rate. Again, sequence analysis in GenBank disclosed a complex repeat motif with several mononucleotide runs flanking the core tetranucleotide repeat. Very similar observations were obtained in the complex dinucleotide repeats; TP53, for example, showed an (A),A run near the (CA), repeat.

Definition of MSI Tumors: Clinical and Diagnostic Implications. We found that 15 tumors displayed MSI of more than 20% of the 31 loci tested (high MSI tumors; Fig. 2). In 12 carcinomas, the frequency was below 10% with most cases (n = 9), exhibiting only one unequivocal unstable locus (low MSI tumors). Thirty-one tumors did not show unequivocal instability at the 31 tested loci. Interestingly, these three tumor groups differed both with respect to their clinical data as well as their mismatch repair gene status. One-half of the patients with high MSI tumors (8 of 15) fulfilled either the Amsterdam criteria (n = 4), had multiple primary carcinomas of the HNPPC spectrum (patient 38 in Table 2), or had at least one relative suffering from a HNPPC-related cancer (n = 2). Two other patients were of young age (below 45 years). In the low MSI group, only one patient fulfilled the Amsterdam criteria, two had at least one relative with a HNPPC-related carcinoma, and one patient was of young age. In the MSI-negative group, no patient met the Amsterdam criteria, two had a positive family history of HNPPC-related tumors, and one was of young age. In addition, only one of the 15 patients (6.6%) with a high MSI tumor died during the follow-up period, whereas 16.6% (2 of 12) of patients with low MSI tumors and 22.6% (7 of 31) of patients with MSI-negative carcinomas succumbed. Furthermore, a close relationship between mismatch repair gene expression and MSI status was found. Loss of hMSH2 or hMLH1 immunostaining was restricted entirely to the high MSI cases and could be demonstrated in 14 of the 15 cases (8 hMSH2-negative and 6 hMLH1-negative MSI tumors). Direct exon sequence analysis in two of these patients has revealed that they...
Table 1 Characteristics of the microsatellite markers analyzed

<table>
<thead>
<tr>
<th>Name (locus)</th>
<th>Chrom. loc.</th>
<th>Repeat motif</th>
<th>Primer sequence (5' to 3')</th>
<th>PCR-Tm°</th>
<th>size (bp)</th>
<th>Reference</th>
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<tr>
<td>BAT-25</td>
<td>4q12</td>
<td>(TTT)(_{10})</td>
<td>TCG CTC CAA AGA ATG GTA</td>
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<td>~90</td>
<td>Papadopoulos et al. (38)</td>
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<td>2p</td>
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<td>TCT CCA TTT TAA CTA GTC CTC</td>
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<td>~80-100</td>
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<td>TTTT.C(T(_{7}))</td>
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<td>S2H10</td>
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<td>S6C10</td>
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<td>D1</td>
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<td>~80-100</td>
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<td>M6411</td>
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<td>157-171</td>
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<td>(D17S261)</td>
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<td>101-113</td>
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<td>150-160</td>
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<td>APC</td>
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<td>142-156</td>
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<td>GCA TAG TCG GTA ACC AT</td>
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<td>~161</td>
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<td>~185</td>
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<td>~159</td>
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<td>Jones and Nakamura (48)</td>
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<td>52°C</td>
<td>~172</td>
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</table>

a Chrom. loc., chromosomal location; NA, not available.
b Nonrepetitive nucleotides are indicated as dots.
c PCR-Tm, PCR annealing temperature.
d A. Hartmann, personal communication.
* Reference Genetics, Huntsville, AL.
Fig. 1. Examples of 25 different microsatellite loci from paired normal and tumor tissue analyzed by silver-stained polyacrylamide/urea sequencing gels. A, mononucleotide repeats. 1, BAT25; 2, BAT26; 3, BAT40. B, noncomplex (“pure”) dinucleotide repeats. 1, DSS346; 2, D13S175; 3, D3S1283; 4, D18S34; 5, D10S89; 6, D17S261. C, complex dinucleotide repeats. 1, D17S250; 2, D10S197; 3, D11S1318; 4, D11S904; 5, D18S59; 6, D2S123; 7, D9S171; 8, D18S58; 9, TP53FLC15. D, trinucleotide repeats. 1, AR; 2, TBP. E, tetranucleotide repeats. 1, Mycll; 2, HPRT 11; 3, RB. F, pentanucleotide repeats. 1, TP53; 2, FMR2.

harbor hMSH2 germline mutations (patients 28 and 39, Table 2), and sequence analysis of the remaining high MSI cases is ongoing.

Comparison of the results obtained with the initial five-primer set used to select the 58 colorectal cancer cases from the ongoing 200-case prospective study suggests that the panel of microsatellite primers used for diagnosis may affect the identification of MSI. For example, of the 14 high MSI cases, 12 remained high MSI, but 2 were subsequently classified as low MSI; of the 9 originally classified as indefinite MSI, 1 was subsequently found to be high MSI, 2 were called low MSI, and 6 were subsequently catalogued as MSS; finally, of the 35 cases diagnosed as MSS with the original microsatellite markers, 2 were subsequently found to be high MSI and 8 were found to be low MSI. It was from this recategorized diagnosis that the enhanced correlation of MSI status with loss of expression of hMSH2 or hMLH1 was observed.

**MSI Tumor Diagnosis Using a Selected Primer Panel.** Our data clearly indicate that: (a) the frequency of MSI detection varies markedly with respect to the loci tested; and (b) highly unstable tumors form a distinct entity with respect to family history, to mismatch repair gene status, and to prognosis. Based on these data, a panel of microsatellite markers could be defined that had the highest sensitivity and specificity in the detection of the highly unstable tumors. Of the 26 microsatellite primer pairs that detected at least some instabilities, about one-half recognized selectively the high MSI tumors (n = 15). Another 11 primer pairs additionally identified some of the low MSI cases (Fig. 3). With respect to robust amplification, easy interpretation, sensitivity, and specificity, a panel of five first-choice primers were selected; BAT26, BAT40, and Mfd15 appeared to be most specific in detecting the high MSI tumors, whereas APC and D2S123 showed the highest sensitivity, recognizing both low and high MSI cases. All of our high MSI tumor cases would have been diagnosed using these primers and the more restricted definition of at least 40% instability as the litmus test. The enhanced specificity and sensitivity of this microsatellite marker panel allowed a refined definition of MSI from our original requirement of 20% instabilities to the more restrictive 40% instabilities. In addition, we would suggest the use of an additional five microsatellite markers if only one of five initial microsatellite markers was found to be unstable in the first analysis. These are BAT25, D10S197, D18S58, D18S59, and MYCL1. However, the litmus of at least 40% instability would still be used to define MSI. The use of these latter microsatellite primer pairs may help to further define the low instability cases as a distinct tumor type. Alternatively, the same result for the high MSI tumors would have been obtained if only BAT26 and MFD15 had been taken into account (25). Although we clearly recommend the use of the entire microsatellite marker panel, the use of these two markers may provide an efficient, low-cost, initial screen.

**DISCUSSION**

The present study confirms and extends the observation that microsatellite instability is a nonrandom event in colorectal cancer...
with microsatellite loci containing mononucleotide repeat motifs, particularly of (A)n type, being most often affected. Moreover, we show that complex dinucleotide markers that detect repetitive segments in addition to the internal (CA)n run are more unstable than noncomplex sequences containing (CA)n repeats exclusively. At least in colorectal cancers, tri-, tetra-, and pentanucleotide repeats are significantly less altered than mono- and complex dinucleotide repeats. From a clinical diagnostic point of view, a panel of five microsatellite markers is recommended as first-choice markers for routine praxis and a second five primers recommended to further define the low MSI tumors. Parameters critical for primer selection were reliability of interpretation as well as sensitivity and specificity in the detection of tumors that express the “true” MSI phenotype.

Assessment of microsatellite instability has become an important tool in tumor molecular pathology. MSI and MSS appear to characterize two different pathways of carcinogenesis (26). Furthermore, patients that present with colorectal MSI carcinomas have a better prognosis than MSS tumors (2, 27). It is now widely accepted that MSI assessment should be added as a distinct criterion to refine the diagnosis of HNPCC. Finally, the status of the mismatch repair system may be important in predicting tumor response to clinical therapy because it appears that mismatch repair-deficient cells are resistant to the chemotherapeutic agents cisplatinum, 5-fluorouracil, and mephalan, while being more sen-

Fig. 2. Frequency of microsatellite instability in 58 tested colorectal cancers. X-axis, the number of unstable loci. Y-axis, the number of tumors with the respective level of MSI.
DIAGNOSTIC MICROSATELLITE ANALYSIS

Repeat type | Locus | low MSI tumors | high MSI tumors
--- | --- | --- | ---
Mono | BAT25, BAT26, BAT40, 52H10, 50C10, D1 | | |
| | | | |
Di non-complex | APC, D13S175, D3S1283, Mfd26, Mfd28, Mfd41 | | |
| | | | |
Di complex | D1d15, D10S197, D11S1318, D11S904, O18S69, D2S123, D6S171, O18S88, TP53PCR | | |
| | | | |
Tri | AR, TBP, SR | | |
| | | | |
Tetra | MYCL1, RB, REN, HPRT I, HPRT II | | |
| | | | |
Penta | TP53Alu, FMR2 | | |

Fig. 3. Sensitivity and specificity of 31 analyzed microsatellite markers. □, number of detected low MSI tumors; □, number of detected high MSI tumors. Markers labeled with • are recommended as first-choice primers. Markers labeled with ○ are recommended as second-choice primers.

sitive to gamma-radiation (20). With this in mind, it is extremely important to reliably assess the MSI status of colorectal tumors in a diagnostic clinical setting. Unfortunately, no generally accepted rules exist regarding the number and type of microsatellites that should be examined. Moreover, the definition of MSI has not been determined (21). Here, we have studied a series of 58 colorectal cancers at 31 microsatellite loci that comprised different simple repeat motifs, including mono-, di-, tri-, tetra-, and pentanucleotide repeats. The tumors studied were selected out of a prospective study on colorectal cancer solely on the basis of an initial determination of MSI status at five dinucleotide loci. Detailed clinical information about patients family history as well as patients outcome was then determined. All tumors were immunohistologically examined for the expression of the two mismatch repair proteins hMSH2 and hMLH1.

Although several studies have already shown that particular repeat loci are especially prone to instability in colorectal cancer, this is the first study in which a broad spectrum of repeat motifs comprising the most relevant types of microsatellites have been systematically examined. Our data clearly show that certain microsatellite markers are particularly susceptible to instability. Mononucleotide repeats were most often affected by MSI, which was followed by dinucleotide repeats with complex repeat motifs. Tri-, tetra-, and pentanucleotide repeats showed the lowest instability frequency. The susceptibility of mononucleotides to instability in MSI tumors has initially been described by Ionov et al. (1), where replication errors occurred most frequently in Alu repeats that had undergone deletions in their poly(A) tails. The (A)n tracts located in an intron of the c-kit oncogene (BAT25), in intron 5 of hMSH2 gene (BAT26), and in an intron of the 3β-hydroxysteroid dehydrogenase gene (BAT40) appear to be highly affected by instability (28). The frequency of MSI at these long [greater than (A)34] repeats appears to be almost twice as high as for shorter mononucleotide repeats. However, these shorter mononucleotide repeats are of particular interest for carcinogenesis because they occur in the coding sequences of important growth regulatory genes (transforming growth factor β1 receptor type II and insulin-like growth factor type II receptor), apoptosis genes (BAX), and immune surveillance genes (14, 15, 28, 29). Mutations in short mononucleotides stretches of hMSH3 [(A)8; 39%] and hMSH6 [(G)8; 30%] genes occur less frequently (15, 30), whereas mutations in short mononucleotide repeats in genes neither involved in proliferation nor apoptosis nor mutator genes occur significantly less frequently or not at all (15). This observation suggests that there is a positive growth advantage in MSI tumors for the accumulation of altered (A)n tracts located within exons of genes involved in tumorigenesis.

Although these data confirm the susceptibility of mononucleotide repeats to frameshift mutations in the MSI tumor pathway, the frequency of mutations in each of the short mononucleotide tracts is generally lower (ranging from 10 to 50%) than in long (A)n runs, where we found band shifts in 60–87% of our MSI tumors. This observation is similar to Jin et al. (31), who found that repeats with large sequence length may be more easily accessible for slip-mispairing than short ones. This phenomenon is likely to be influenced by additional sequence structures surrounding the repeats because complex dinucleotides were more often affected than pure dinucleotides.
Our data confirm the observation that BAT26 is perhaps the best marker for MSI assessment in colorectal cancers (25). Although BAT26 was found to be sufficient to confirm the MSI status in nearly all colorectal cancers in this previous study, we found that BAT26 did not detect two of our 15 high MSI tumors. This leads to the question of which tumors should be scored as MSI. Usually the diagnostic criterion of a MSI tumor relies upon the number of unstable loci tested, which has not yet been unambiguously defined. The number of tested microsatellite primers varies from two to more than 100 (reviewed in Ref. 8), and the number of unstable loci required to score a tumor as MSI usually ranges from 1 or 2 loci or is given as a percentage ranging from 10 to 50% (32-34). Thus, the frequency of MSI tumors reported in the literature differs remarkably, indicating an urgent need of standardized MSI criteria.

In this study, we have included further clinical, pathomorphological, and mismatch repair gene data. MSI tumors are distinguished by: (a) a distinct pathomorphological tumor phenotype (11, 20, 23); (b) a favorable prognosis (27); (c) a close relationship of family history, particularly to the Amsterdam criteria; and (d) by the loss of hMLH1 and hMSH2 repair gene expression (35). In the present study, 15 tumors that exhibited at least 20% unstable microsatellite loci of the 31 tested were scored as MSI tumors. Most of these patients had either a positive family history of HNPPC (n = 4) or had a favorable prognosis (n = 14 alive after 29 months median follow-up time). But most strikingly, a close relationship between immunostaining and the frequency of MSI was found. Fourteen of 15 tumors with MSI in at least 20% of the tested loci showed either a loss of the hMSH2 (n = 8) or of the hMLH1 mismatch repair proteins (n = 6). In contrast, no tumor with low MSI frequency (including nine with only one unstable locus) showed a loss of hMSH2 or hMLH1 expression by immunohistochemistry. These results are qualitatively similar to Thibodeau et al. (35). The strong correlation of high MSI with loss of expression of either hMSH2 or hMLH1 has been further confirmed in a nearly complete analysis of a large colorectal tumor cohort. We have completed analysis of 103 of 200 total colorectal cancer cases, including all of the high and low MSI tumors. We have found 25 cases that displayed high MSI, and 23 of these had lost expression of either hMSH2 (12 cases, 48%) or hMLH1 (11 cases, 44%). The underlying mutations responsible for this loss of expression are under study. It still needs to be assessed whether there are hMSH2 or hMLH1 mutations in the two high MSI cases that showed no detectable loss of hMSH2 or hMLH1 protein expression, or whether there is altered expression or mutations in one of the other mismatch repair genes, *hMSH3, hMSH6, or hPMS2*. Of the remaining tumors analyzed, 35 cases displayed low MSI, and 43 cases were MSS; in none of these tumors was the expression of hMSH2 or hMLH1 altered.

Based upon these data, a set of primers was chosen being most sensitive and specific in the detection of high MSI. We suggest that a combination of mononucleotide, dinucleotide, and trinucleotide repeats and complex dinucleotide repeats is able to detect all MSI tumors reliably. Furthermore, at least 40% of these microsatellite markers should display instability for the tumor to be classified as MSI. We recommend a 10-primer set for examining MSI. First, *BAT26, BAT40, Mfd15, D2S123, and APC* should be examined. We found that this primer set would have detected 100% of the high MSI tumors in our cohort. If a single microsatellite primer pair appears to display instability (<40%), then *BAT25, D10S197, D18S88, D18S69*, and *MYCL1 microsatellite sequences* should be examined for instability. However, the classification of MSI would still be limited to the finding of at least 40% unstable loci. When each criteria, specificity, sensitivity, and unequivocal interpretation of band patterns were considered, the simplest combination was achieved by the use of one mononucleotide marker, BAT26, and one complex dinucleotide marker, Mfd15. Either of these two markers was unambiguously altered in each MSI tumor examined.

Although we are presently studying this primer set in multiplex PCR on an automated sequencer, to our knowledge, the detection method of PCR products is not critical. According to a multicenter MSI study performed recently, unstable tumors were detected with excellent agreement between eight laboratories throughout Germany (despite the use of four different detection methods; Ref. 36). Most recently, another strategy to identify MSI tumors, a variant of AP-PCR, so-called Alu/AP-PCR, has been proposed as a more sensitive detection method of MSI tumors (37). Although more sensitive than the original AP-PCR method, this technique is not applicable to formalin-fixed and paraffin-embedded tissue and is, therefore, not suited for routine diagnosis.

In summary, we have described a reliable diagnostic strategy for MSI assessment. In combination with immunohistochemistry, this strategy has the potential to diagnose more than 90% of tumors that involve alterations of hMSH2 and hMLH1.

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