Low-Level Microsatellite Instability in Most Colorectal Carcinomas


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ABSTRACT

Twelve to 16% of colorectal cancers (CRCs) display a high degree of microsatellite instability (MSI-H), whereas most are believed to be microsatellite stable (MSS). The existence of a low degree of instability (MSI-L) group has also been proposed. By using the Bethesda panel of microsatellite markers, the microsatellite instability (MSI) status of CRCs can be determined. This set is recommended to distinguish between MSI-H and MSI-L/MSS. No definition for MSI-L has emerged. Most reports on MSI-L rely on the Bethesda panel, using 5–15 markers. Tumors with more than 30% MSI are designated as MSI-H, but the lower limit for MSI-L is ambiguous. We hypothesized that if many markers are studied, almost all CRCs would show some MSI. It would be necessary to establish a cutoff level for MSI-L by showing that, above this cutoff level, tumors display molecular and/or clinical features different from those under the cutoff level. To perform this task, we analyzed 90 BAT26 stable CRC samples with 377 markers. MSI at 1–11 loci was observed in 71 (79%) of the 90 cases. K-RAS mutation, loss of heterozygosity, and MSH1 and MGMT hypermethylation analyses were performed, as well as clinical features being scrutinized, to examine possible differences between MSI-L and MSS tumors using all of the possible cutoff levels for MSI-L. Convincing differences between putative MSI-L and MSS groups were not observed. Our results show that the sensitivity of a typically used marker number to detect MSI-L is very low, and they suggest that MSS and MSI-L tumors have a common molecular background.

INTRODUCTION

Prominent MSI is a phenomenon caused by a failure of the DNA MMR system to repair errors that occur during DNA replication. MMR deficiency causes an accelerated accumulation of single nucleotide mutations and, particularly, alterations in the length of short, repeated DNA sequences, i.e., microsatellites. MSI characterizes HNPCC but is also found in 12–16% of unselected CRCs (1–3). CRCs are usually divided into two subgroups depending on the absence or presence of MSI; tumors that display MSI-H (typically more than 30% of studied loci altered), and tumors that appear to be MSS (4, 5). It has been proposed that a third group of tumors characterized by MSI-L exists (4, 5). The recommended method to distinguish the MSI-H and MSI-L/MSS subgroups is to study tumor and respective normal tissue DNA using a panel of five microsatellite markers: BAT25, BAT26, D2S123, D5S346, and D17S250, known as the Bethesda panel (6). If no microsatellite mutations are observed, the tumor is typically designated MSS, although MSI-L phenotype cannot be excluded. If two or more of the marker loci display instability, the tumor is considered MSI-H. If one marker displays a mutation, additional markers should be analyzed to distinguish between MSI-L and MSI-H; if more than 30% of the markers display MSI, the tumor is designated MSI-H, and the remaining cases fall into the MSI-L category (6).

Most CRCs are believed to be MSS, and MSI-H accounts for 12–16% of cases (1–3). MSI-L tumors determined as outlined above, using 5–15 markers, seem to be more rare, but a wide range (4 to 33% of all lesions) of estimates have been published (7, 8). If a large number of microsatellite markers are used, it may be that all CRCs would exhibit some level of MSI (6). The method proposed for the detection of MSI-H cancers seems to be functional because clear clinical and molecular differences between MSS/MSI-L and MSI-H cancers are evident (9–12). However, relatively little attention has been paid to the definition of MSI-L.

Molecular subclassification of CRC based on the absence or presence of MSI has important clinical implications. Biological and clinical features, including prognosis, of MSI-H and MSS/MSI-L tumors are strikingly different (9–12). MSI-L and MSS tumors display many more similarities. Because the baseline mutation rate for microsatellites is not precisely known, and the clinical features of these two groups are similar, combining these two groups seems feasible (6). However, although some studies have not found evidence that MSI-L and MSS tumors should be divided into different groups (11, 13–17), other reports do propose such a subclassification (8, 18–22). An excess of K-RAS mutations in MSI-L tumors versus MSS tumors has been observed (8). The same work also showed that 5q LOH is less frequent in a MSI-L group. Reduced BCL-2 expression (21), higher apoptotic activity, and lymphocytic infiltration may be associated with MSI-L (22). It has also been reported that MGMT gene promoter methylation correlates with K-RAS mutations in MSI-L tumors (18).

The hypothesis that if enough markers are studied, almost all CRCs would show some degree of MSI (6) seems reasonable. If this were correct, it would be necessary to establish a cutoff level for MSI-L by showing that, above this cutoff level, tumors display molecular and/or clinical features different from those under the cutoff level. To perform this task, we analyzed 90 BAT26 stable CRC samples with 377 markers, and examined the mutative molecular and clinical differences between the two groups using all of the possible cutoff levels for MSI-L.

MATERIALS AND METHODS

Specimens. Ninety CRC samples were selected from a population-based series of 1044 CRCs, none of which were designated MSI-H in previous works.
when analyzed with poly(A) marker BAT26, a robust marker for MSI-H detection (7, 23, 24). This selection was performed to allow focus on MSI-L and MSS samples. Either normal mucosa or normal blood had been used as a source of normal DNA. All of the cancer samples had displayed >50% carcinoma tissue. Sixty-three of the cases were sporadic, and 27 had one first-degree relative with CRC.

**Microsatellite Analyses.** The MSI status of the 90 samples was subsequently examined using the full basic set of five Bethesda markers (BAT25, BAT26, D2S123, DSS346, and D17S250). PCR reactions and primer sequences have been described previously (7). The MSI analysis was continued with eight markers listed in the recommended set for MSI analyses (D18S318, D1S86, D10S517, D1S8175, D18S787, D17S157, and D2S100; Ref. 6). Subsequently, 364 additional CA-repeat markers were analyzed (ABI PRISM Linkage Mapping Set MD-10, P/N 450067; Applied Biosystems (AB) Division, Foster City, CA). PCR products were run in ABI PRISM 377 DNA Sequencer (AB) according to the manufacturer’s instructions, and the collected data were analyzed by GeneScan 3.1 and Genotyper 2.5 software programs (AB). The results were independently visually evaluated by four different individuals (P. Lai, P. Lah, V. L., and L. A. A.). Tumors showing alleles that were not present in corresponding normal tissue were considered MSI positive. In cases with any ambiguity, a previously presented mathematical model known as “RER score” was used to score for MSI (25).

Locus-specific LOH analysis was performed using five additional microsatellite markers on chromosomes 5q (D5S318 and DSS346), 17p (TP53) and 18q (D18S1156 and D18S363). LOH was scored if an allele was decreased by more than 40% (25).

To examine for possible topographic mutation heterogeneity of the observed MSI shifts, stained microscopic sections from three MSI-L cancers were subdivided by microdissection into 6–10 small (3-mm; Ref. 2) subregions. The sizes of the microsatellite alleles present in each subregion were determined by electrophoresis with 6% acrylamide sequencing gels after incorporating [33P]dCTP during 38 PCR cycles as described previously (26). In addition, DNA from whole tumors was diluted to essentially single molecules before PCR to determine whether intratumor microsatellite mutation heterogeneity (shifts of multiple sizes) was present (27). Approximately 10 alleles were examined at each locus.

**K-RAS Mutation Analysis.** K-RAS codons 12, 13, and 61 were screened for mutations. Exons 1 (codons 12 and 13) and 2 (codon 61) were amplified as described previously (28). Direct sequencing of the PCR products was performed using cycle sequencing with Big Dye Terminator kit (AB), and reactions were run on ABI 3100 capillary sequencer (AB) according to manufacturer’s instructions.

**Methylation Analysis.** DNA methylation patterns in the CpG island of MLH1 and MGMT were determined by chemical modification of unmethylated, but not methylated, cytosines to uracil, and subsequent PCR using primers specific for either the methylated or the modified unmethylated DNA (29–31). The CRC cell line SW48 was used as the positive control for methylated alleles of MLH1 and MGMT, and DNA from normal lymphocytes was used as negative control. Ten μl of each PCR reaction were directly loaded onto nondenaturing 6% polyacrylamide gels, stained with ethidium bromide, and visualized under UV illumination.

**Statistical Analysis.** To assess whether the distribution of MSI detected in the cancers was different from that expected by random chance, 100,000 bootstrap replicates of the data were simulated using the probabilities of MSI derived from the observed frequency. The statistical significance was derived from the number of times the observed distribution was attained.

Clinical and molecular differences between the putative MSS and MSI-L groups were assessed using Fisher’s exact test or the maximized \( \chi^2 \) test (32). The relationship between the frequency of MSI and variables was evaluated by multiple regression analysis. ORs and associated 95% CIs were obtained through logistic regression. All of the standard statistical manipulations were undertaken using the statistical program STATA version 6.0 (Stata Corporation, College Station, TX). Maximized \( \chi^2 \) scores were obtained by simulation using the program CLUMP (32). To compare the ORs of K-RAS mutations associated with MSI-L in published reports, pooled estimates of risk were obtained using standard calculations for the weighted-average of the logarithm of ORs (33). A \( P < 0.05 \) was considered significant.

## RESULTS

**MSI Analysis.** Nineteen of the 90 CRCs (21%) did not display any MSI when analyzed with 377 microsatellite markers. The total number of successfully analyzed markers per 1 sample pair was at least 273. In the initial analysis with five basic Bethesda markers, 6 (7%) of the 90 tumors showed MSI. The addition of eight markers listed in the Bethesda reference panel resulted in 9 (10%) of 90 tumors displaying MSI. After the completion of an additional 364 markers, 71 (79%) of 90 had displayed some evidence of MSI (Fig. 1). The number of mutated loci varied from 1 to 11 (Fig. 2). The distribution of MSI among the cancers analyzed was significantly different from that expected if random (\( P < 10^{-4} \)) and was in large part ascribable to an excess of cancers displaying no MSI. This observation provides a strong rationale for setting a MSS cutoff level for comparisons to 1 in this study (that is, detection of 1 or more MSI positive loci of 377

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*The list of the markers is available from the authors on request.*

Fig. 1. Examples of MSI. For each marker: normal DNA (N, upper graph); tumor DNA (T, lower graph). Marker D3S1580, an example of a rare, more dramatic MSI pattern; marker D1S2841, an example of a frequently seen addition of one CA. D2S151, an example in which the RER score was calculated to confirm the MSI status (RER score, 25%).

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was interpreted as MSI-L). However, all 11 possible cutoff levels (Fig. 2; Tables 1 and 2) were scrutinized for significant associations. Only dinucleotide markers displayed instability. The nine lesions showing MSI after an analysis of the first 13 Bethesda markers displayed zero to eight microsatellite mutations in the additional set of 364 markers. The most sensitive among the 377 markers were D6S441 and D10S217. These were able to detect six and five positive lesions, respectively.

Topographical tumor distributions of the microsatellite mutations were usually homogeneous because mutations were focally present with only 2 of 13 microsatellite loci examined from three MSI-L cancers. This topographical heterogeneity was present at two of six loci from a single cancer. However, it is uncertain whether this tumor heterogeneity represents a lack of mutation or a loss of mutant alleles because heterogeneity with respect to LOH was also observed. Mutation size heterogeneity at 22 microsatellite loci was not observed when tumor DNA was diluted before PCR, which indicates only a single somatic mutation was present throughout the tumor.

**Comparison between Putative MSI-L and MSS Groups.** There was no significant relationship between Dukes classification and MSI-status using all of the eleven possible cutoff levels. The only statistically significant relationship detected between grade and MSI-status was obtained if MSS was defined by <3 mutations as a cutoff level ($P = 0.01$ using 0 as the cutoff). Any possible relationship was not, however, reflected in a relationship between microsatellite mutation frequency and grade that was not significant ($P = 0.3$). There was no evidence for a relationship between MSI-L status and either family history or site of cancer using cutoff levels or through an analysis of the relationship of each to mutation frequency.

The lack of widespread allelic or topographical microsatellite mutation heterogeneity coupled with no significant evidence for a relationship between frequency of mutation and stage or grade suggests that most microsatellite mutations were acquired before final clonal expansion. To further investigate the interval before clonal expansion, the relative frequency of microsatellite mutation was plotted with respect to patient age at tumor removal (Fig. 3). There was a significant relationship between age and the frequency of MSI ($P = 0.012$). Furthermore, there was no evidence for an interaction between age and either stage or grade. On the basis of the regression coefficient, the implied rate of mutation is $4 \times 10^{-7} (95\% \text{ CI}, 1-8 \times 10^{-7})$ under a simple stochastic model of one division per day (Fig. 3).

The relationship between MSI-status and K-RAS-mutation; MGMT and MLH1-methylation; and 5q, 17p, and 18q LOH was evaluated to investigate whether MSI-L cancers have a distinctive genetic phenotype from MSS lesions.

Twenty-nine tumors had K-RAS mutations. There was no evidence that the frequency of K-RAS mutations is different between MSS and MSI-L cancers when data is stratified according to different cutoff levels. Using a cutoff of zero microsatellite mutations to define MSS, there was no statistically significant relationship detected between grade and MSI-status using all of the eleven possible cutoff levels.

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<table>
<thead>
<tr>
<th>No. of MSI-positive loci</th>
<th>K-RAS mutation</th>
<th>$M$</th>
<th>$F$</th>
<th>Average age (range)</th>
<th>Familial</th>
<th>Sporadic</th>
<th>Proximal</th>
<th>Distal</th>
<th>Grade</th>
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<td>0 (n = 19)</td>
<td>7/19 (37%)</td>
<td>1</td>
<td>2</td>
<td>60.3 (38–78)</td>
<td>5</td>
<td>14</td>
<td>6</td>
<td>6/5/2</td>
<td>2</td>
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<tr>
<td>1 (n = 9)</td>
<td>3/9 (33%)</td>
<td>1</td>
<td>1</td>
<td>53.6 (38–72)</td>
<td>1</td>
<td>1</td>
<td>3</td>
<td>3/2/1</td>
<td>2.5</td>
</tr>
<tr>
<td>2 (n = 15)</td>
<td>4/15 (27%)</td>
<td>6</td>
<td>9</td>
<td>64.4 (40–89)</td>
<td>6</td>
<td>9</td>
<td>3</td>
<td>12</td>
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<tr>
<td>3 (n = 11)</td>
<td>5/11 (45%)</td>
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<td>2</td>
<td>64.5 (46–73)</td>
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<td>9</td>
<td>4</td>
<td>7</td>
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<td>12</td>
<td>60.6 (50–72)</td>
<td>6.1</td>
<td>10</td>
<td>2</td>
<td>10</td>
<td>6.9</td>
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<td>1/7 (14%)</td>
<td>18</td>
<td>22</td>
<td>65.4 (41–79)</td>
<td>2.5</td>
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<tr>
<td>6 (n = 7)</td>
<td>0/7 (0%)</td>
<td>21</td>
<td>22</td>
<td>68.3 (58–84)</td>
<td>4.3</td>
<td>3</td>
<td>2</td>
<td>5</td>
<td>3.3</td>
</tr>
<tr>
<td>7 (n = 5)</td>
<td>3/5 (60%)</td>
<td>22</td>
<td>25</td>
<td>70.5 (58–73)</td>
<td>1</td>
<td>4</td>
<td>3</td>
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<td>30</td>
<td>72.9 (57–67)</td>
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<td>9 (n = 3)</td>
<td>2/3 (67%)</td>
<td>34</td>
<td>37</td>
<td>72.3 (64–87)</td>
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<td>38</td>
<td>62.6 (52–58)</td>
<td>1</td>
<td>0</td>
<td>1</td>
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</tr>
<tr>
<td>11 (n = 1)</td>
<td>0/1 (0%)</td>
<td>38</td>
<td>41</td>
<td>62.5 (38–58)</td>
<td>1</td>
<td>0</td>
<td>1</td>
<td>0</td>
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</tbody>
</table>

**Table 1.** MSI-L IN MOST COLORECTAL CARCINOMAS

Comparison between Putative MSI-L and MSS Groups. There was no significant relationship between Dukes classification and MSI status using all of the eleven possible cutoff levels. The only statistically significant relationship detected between grade and MSI-status was obtained if MSS was defined by <3 mutations as a cutoff level ($P = 0.01$ using 0 as the cutoff). Any possible relationship was not, however, reflected in a relationship between microsatellite mutation frequency and grade that was not significant ($P = 0.3$). There was no evidence for a relationship between MSI-L status and either family history or site of cancer using cutoff levels or through an analysis of the relationship of each to mutation frequency.

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K-RAS mutations were detected in 37% of MSS (95% CI, 16–62%) and 31% of MSI-L lesions (95% CI, 20–49%; P = 0.26; Table 1). The OR was 0.8 (95% CI, 0.3–2.2). There was no evidence for an association between K-RAS mutation and microsatellite mutation frequency (P = 0.78).

The MLH1 promoter was methylated in 5% of MSS tumors (95% CI, 0.1–26%) and in 10% of MSI-L tumors (95% CI, 4–19%). This difference was not significant (P = 1.0). Similarly there was no significant difference in the frequency of MGMT promoter methylation between the two groups; 11% in MSS cases (1–33%) and in 27% of MSI-L cases (95% CI, 17–39%), respectively (P = 0.22; Table 1). There was no evidence for a relationship between MLH1 or MGMT promoter methylation and frequency of microsatellite mutations (P = 0.44 and 0.74, respectively).

LOH at chromosome 5q was detected in 16% of MSS cancers (95% CI, 3–40%) compared with 36% of MSI-L cancers (95% CI, 25–49%; P = 0.10). LOH at chromosome 17p was detected in 38% of MSS cancers (95% CI, 15–64%) compared with 53% of MSI-L cancers (95% CI, 40–66%; P = 0.40). LOH at chromosome 18q was detected in 60% of MSS cancers (95% CI, 26–88%) compared with 27% of MSI-L cancers (95% CI, 4–71%; P = 1.0).

In the MSI-L group, there was no correlation between K-RAS mutation and MGMT methylation status (P = 1.0). Simultaneous K-RAS mutation and MGMT methylation was seen in 6 (32%) of 19 MSI-L tumors. In MGMT-unmethylated MSI-L tumors, K-RAS was mutated in 16 (31%) of 52 tumors. K-RAS mutations were not observed with MLH1 promoter methylation. In four MSI-L tumors, MGMT and MLH1 were both methylated (Table 1).

**DISCUSSION**

Molecular classification of CRC based on MSI status has important potential in patient management. It has been convincingly demonstrated that the origin of MSI-H tumors is different from MSS tumors. Some studies report that MSI-L tumors have distinct molecular features from MSS CRCs, such as high frequency of K-RAS mutations, low frequency of 5q LOH (8), reduced BCL-2 expression (21), higher apoptotic activity, and lymphocytic infiltration (22). Nevertheless, in general, most of the clinical and molecular features in MSI-L cancers are similar to those of MSS cancers (9, 10, 11, 13–17), and these two groups can be distinguished neither clinically nor morphologically.

It is, therefore, relevant to determine the background and significance of MSI-L. Although it is conceivable that some rare lesions with a particular molecular background, such as fast-growing tumors with MSH2 or MLH1 deficiency, or mutations only subtly affecting the MMR system (e.g., some missense mutations of MLH1 and MSH2, mutations in MSH6 and other minor MMR genes), can lead to MSI-L phenotype, the mechanisms underlying the majority of these lesions remain to be clarified.

To clarify the nature of the MSI-L lesions one first has to define MSI-L. The results reported here show that the number of microsatellite markers typically used for MSI-L detection is not adequate. A small number of markers will identify a subset of MSI-L, but the majority of tumors with a comparable degree of MSI are not detected. Analysis of as many as 13 microsatellite markers listed in the Bethesda panel proved to be a very insensitive method of detecting MSI-L tumors, leaving in our data set nearly 70% of such lesions undetected, as demonstrated by a subsequent analysis of 364 microsatellite markers. If large microsatellite numbers are used, we hypothesize that 100% of CRCs display a MSI-L phenotype. It would be necessary to establish a cutoff level for MSI-L by showing that, above this cutoff level, tumors display molecular and/or clinical features different from those under the cutoff level.

In the present work, a set of 377 microsatellite markers displayed MSI at 0–11 loci in the 90 study sample pairs. When examining all of the 11 possible cutoff levels to separate MSI-L and MSS in our data set, we did not find any convincing molecular or clinical differences between the putative MSS and MSI-L groups in terms of family history, sex, site, stage, grade, K-RAS mutations, MGMT promoter hypermethylation, MLH1 promoter hypermethylation, and LOH at APC, p53, and DCC-SSAD4 loci. We, however, acknowledge that our study is insufficiently powered to demonstrate small differences (i.e., 2- to 3-fold) in the promoter methylation of MGMT and MLH1, or K-RAS mutation.

Although no significant differences were apparent between MSS and MSI-L groups, the distribution of microsatellite mutations among different lesions was not random, i.e., more microsatellite mutations accumulated in some cases. Microdissection experiments failed to demonstrate widespread genetic heterogeneity in the microsatellite mutation patterns, implying that most mutations had occurred before the final clonal expansion. Consistent with this result, cancers from older patients tended to have higher numbers of microsatellite mutations (Fig. 3). Interestingly, mutation rates compatible with our data are similar to the median rate of 1.3 × 10⁻⁷ mutations per division observed in normal human fibroblasts (34), consistent with a life-long accumulation of microsatellite mutations in MMR-proficient progenitors. This supports a hypothesis that MSS and MSI-L tumors have a common molecular background with observed instability differences reflecting numbers of cell divisions and stochastic mutation.

After failing to show a convincing difference between MSI-L and MSS CRCs, we evaluated the previously published data on K-RAS mutations in MSI-L CRCs. Whereas Konishi et al. (19), reported a higher frequency of K-RAS mutations in MSI-L cancers (50%) compared with MSS cancer (30%), the difference was not significant (P = 0.13). Jass et al. (8) reported that MSI-L cancers were more likely to display K-RAS mutations than were MSS cancers, because the frequency of mutations was 43% compared with 30%, respectively. Ward et al. (11) recently reported an identical frequency of K-RAS mutations in MSS and MSI-L cancers (29%) and Gerbert et al. (17) reported the frequencies of K-RAS mutation as 29% and 35% in MSI-L and MSS CRCs, respectively. If each study is effectively detecting the same relationship, combining the data from these four published studies does not provide significant support for the notion that the frequency of K-RAS mutations is different between MSI-L and MSS cancers (OR, 1.5; 95% CI, 0.9–2.5; P = 0.8).
The study calls for two important conclusions. First, the commonly used microsatellite marker number, 5–15, to detect MSI-L is inadequate, failing to detect the majority of lesions displaying a similar degree of MSI-L. This must have hampered comparisons between MSI-L and MSS groups in previous studies. Second, it was not possible to propose any number of microsatellite markers and MSI cutoff levels to be used, because even an extensive genotyping effort failed to demonstrate a fundamental difference between the MSI-L and supposedly MSS lesions. Until such a difference can be convincingly demonstrated, the subdivision of CRCs to MSI-L and MSS subgroups does not seem justified.

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