High Frequency of Low-Level Microsatellite Instability in Early Colorectal Cancer

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Abstract

Molecular events in early colorectal cancers (CRCs) have not been well elucidated because of the low incidence of early CRCs in clinical practice. Therefore, we studied 104 sporadic early CRCs with invasion limited to submucosa compared with 116 advanced CRCs. Loss of heterozygosity as well as microsatellite instability (MSI) status was examined. A significantly high frequency of low-level MSI (MSI-L) phenotype was detected in early CRCs (51.0%) compared with advanced CRCs (25.9%; P = 0.0001). In early and advanced CRCs, samples with MSI-L phenotype differed from microsatellite stable (MSS) phenotype with respect to loss of heterozygosity at 1p32 and 8p12–22. MSI-L is a frequent genetic event in early CRCs and may be a novel pathway in colorectal carcinogenesis distinct from both MSI-H and MSS.

Introduction

CRC is a significant cause of morbidity and mortality in industrialized societies. The pathological definition of early invasive CRC is invasion into, but not beyond, the submucosa (1). Subsequent spread into the muscularis propria defines advanced CRC (1). Early-stage CRC often escapes diagnosis in clinical practice, because it is asymptomatic (2). Recent advances in colonoscopic surveillance facilitate detection of early CRCs, and diagnosis of early CRC is no longer infrequent (3, 4). The study of early CRC is relevant to understanding the development of CRC, yet the molecular characteristics of early CRC have not been established. The place of early CRC in the poly-p-cancer sequence must also be considered (3–5).

In sporadic CRC, two MSI phenotypes have been described: MSI-L (MSI at <30–40% of the loci) and MSI-H (MSI at ≥30–40% of the loci; Refs. 6–9). The majority of sporadic CRCs do not show the MSI phenotype and is described as MSS (7, 10). The MSI-H phenotype, which is also observed in HNPCC, accounts for ~15% of sporadic CRCs and results from mutational inactivation of the DNA MMR genes hMLH1 or hMLH2 in HNPCC (9–12) and usually through epigenetic inactivation of hMLHI in sporadic cases (9, 13). The MSI-H phenotype is associated with distinct clinicopathological features, notably proximal tumor site, high grade, diploidy, and favorable prognosis (7, 9, 14). The MSI-L phenotype, on the other hand, is not associated with distinct clinicopathological features or altered hMLH1 or hMSH2 expression (6–8). Currently, the biological basis of the MSI-L phenotype is unknown (7, 15, 16). This phenotype could result from defects in MMR proteins other than hMSH2 and hMLH1 or from defects in genes other than those involved in MMR. MSI-L may also represent a “background” level of genetic instability that is present to some degree in all tumors (15).

In this study, we have used the technique of microdissection to achieve a detailed examination of the genetic alterations in early CRC with invasion limited to the submucosa (7) and have compared these with advanced CRCs to elucidate genetic events occurring during the early phase of invasive malignancy.

Materials and Methods

Tissue Samples. One hundred four early CRCs with submucosal invasion and 116 CRCs with extension into or through the muscularis propria, as well as corresponding normal mucosa, were collected either endoscopically or surgically. We excluded CRCs that occurred in patients with a family history of CRC. CRC with submucosal invasion and some of the advanced CRCs were obtained from formalin-fixed, paraffin-embedded archival material. Serial sections were cut from each paraffin block, and the first section was counterstained with H&E for histopathological diagnosis and as a guide for microdissection. For each tumor sample, an invasive carcinoma region comprising at least 50% malignant tissue was microdissected from 5 to 10 μm unstained serial slides after deparaffinization. The dissected samples, which were ~50 mm², were incubated in 40 μl of lysis buffer [20 mM Tris-HCl (pH 8.0), 1 mM EDTA, 0.5% Tween 20, and 200 μg/ml proteinase K] at 37°C for 24 h and then at 95°C for 15 min to inactivate proteinase K. The DNA for many of the advanced CRCs was obtained from fresh frozen tissue, which was snap frozen immediately after operation.

Microsatellite Analysis. Two mononucleotide markers, 14 dinucleotide markers, and 1 tetranucleotide marker were used to test for MSI or LOH. Mononucleotide marker BAT25 and BAT26 were used for MSI analysis (7). Dinucleotide markers included D2S123 on 2p16; D5S307 on 5q11–13; D5S346 on 5q21–22; D7S480, D7S486, and D7S522 on 7q31; D8S87 on 8p12; D8S254 and D8S258 on 8p22; D17S261 on 17p11–12; D17S960 on 17pter–qter; D17S250 on 17q11–12; D18S35 on 18q21; and D18S58 on 18q22–23 (7, 8, 17, 18). Tetranucleotide marker MYCL, located at 1p32, was used for both LOH and MSI analysis (6, 16). Microsatellite markers selected were those selected from the National Cancer Institute reference set or reported widely and to ensure standardization of results (7). PCR was performed in 50-μl reaction mixtures comprising 3 μl of the 10× DNA sample, 9 μl of GeneReleaser (BioVentures, Inc., Murfreesboro, TN), 0.3 μl each oligonucleotide primer pair (one end-labeled with Texas Red), 200 μM each deoxynucleoside triphosphates, 5 μl of 10× PCR buffer, and 1.25 units of Taq polymerase (AmpliTaqGold; Perkin-Elmer, Foster City, CA). After denaturation in formamide at 95°C for 5 min, the amplified PCR products were electrophoresed on a 6% LongRanger-6.1M urea gel on Hitachi Autosequencer SQ-5500 and analyzed by FRAGLYS version 2 software (Hitachi, Inc., Tokyo). As for MSI, CRCs were classified in three groups: group 1, MSS with no MSI at any of the loci examined; group 2, MSI-L with <40% of the loci examined demonstrating MSI; and group 3, MSI-H with ≥40% of the loci examined demonstrating MSI (6, 7, 14). LOH was determined by ≥50% reduction in the relative intensity of one allelic in the tumor compared with normal.

Immunohistochemical Analysis. Immunohistochemical staining for hMLH1 (clone G168-728, 1 μg/ml; PharMingen, San Diego, CA), hMSH2 (clone FE11, 0.5 μg/ml; Oncogene Science, Cambridge, MA), and hMSH6 (clone 44, 0.5 μg/ml; Transduction Laboratory, Lexington, KY) were per-
formed as described elsewhere (6, 8, 15, 19). The normal staining pattern for hMLH1, hMSH2, and hMSH6 was nuclear. Tumor cells exhibiting an absence of nuclear staining, in the presence of nonneoplastic cells with nuclear staining, were considered to have an abnormal pattern.

**Detection of K-ras Mutation.** Mutation at codon 12 of the K-ras gene was detected by an artificial RFLP/PCR approach as described elsewhere (20).

**Statistical Analysis.** Two-by-two table contingency analyses were performed using a two-tailed Fisher’s exact test or Student’s t test. P < 0.05 was considered to be statistically significant.

**Results and Discussions**

In 116 cases of advanced CRC, 6.9% showed MSI-H, 25.9% showed MSI-L, and the remaining 67.2% were MSS. The frequency of MSI-H (6.7%) in early CRCs was almost the same as advanced CRCs, whereas a high frequency of MSI-L was observed in early CRCs (53 of 104; 51.0%) as compared with advanced CRCs (30 of 116; 25.9%; P = 0.0001; Fig. 1A, Tables 1 and 2).

Associations of MSI status with clinicopathological features in a total of 220 CRCs are shown in Table 1. No statistical difference in clinicopathological factor was detected between MSS and MSI-L cancers, either in early or advanced CRCs. In early CRCs, cancers with MSI-L phenotype were significantly different from MSS cancers with respect to LOH at 1p32 (48.6% versus 18.9%; P = 0.013) and 8p12–22 (71.1% versus 39.0%; P = 0.005; Table 2). In advanced CRCs, the frequency of LOH at 1p32, 2p16, 7q31, 8p12–22, and 17q11 was significantly higher in MSI-L than that in MSS cancers (P = 0.022, 0.001, 0.002, 0.018, and 0.015, respectively; Table 2).

Although numerous molecular studies on different types and sizes of adenomas and advanced CRCs have been conducted and fitted with the classic poly-cancer sequence (5, 12), molecular analysis of early CRCs, and particularly in a large series comprising over 100 cases of early cancer, are uncommon. Molecular analysis of early CRCs is important for achieving a more complete understanding of the steps involved in colorectal carcinogenesis. We therefore analyzed LOH on chromosomes 1p, 2p, 5q, 7q, 8p, 17p, 17q, and 18q as well as the MSI status of 220 sporadic CRCs, including 104 early and 116 advanced CRCs. A high frequency of cancers with the MSI-L phenotype was observed in early CRCs (51.0%) compared with advanced lesions (25.9%; P = 0.0001).

MSI has been identified in tumors from patients with HNPCC as well as in subsets of particular types of sporadic malignancies, including colorectal, endometrial, and stomach (7, 9–11). Two distinct MSI phenotypes have been described in CRCs, MSI-L and MSI-H (6–9). Careful attention has been given to the definition and identification of the MSI-L phenotype in this study. Several groups have shown that the frequency of MSI is distributed bimodally, with a break point at around 30–40% when a panel of markers is used (6, 8).

In the current study, MSI was also distributed bimodally with a break point at ~40% (Fig. 1B). We therefore selected 40% as the breakpoint of MSI-L and MSI-H. However, an analysis using a breakpoint of 30% resulted in the same overall findings. It is likely that the total number and type of markers used may affect the detected frequency of MSI-L (7). We therefore reanalyzed our samples with 40 instead of 17 markers, including those recommended by the National Cancer Institute consensus meeting. Again, there were no differences in the final findings. The minimum five recommended markers were too small to identify all cases with the MSI-L phenotype because no marker is 100% sensitive for MSI-L status (Fig. 1C). The same is true for MSI-H status (Fig. 1C). Thus, our results are unlikely to be explained by the inappropriate use of, or overinterpretation of, microsatellite marker alterations. Immunohistochemical staining with hMLH1 and hMSH2 showed that loss of expression of hMLH1 and hMSH2 was detected only in MSI-H tumors. This indicates that our MSI-L samples are unlikely to include underdiagnosed “true” MSI-H tumors.

The biological basis of MSI-L is not understood (7, 15, 16). This phenotype could result from MMR protein deficiency other than hMLH1 or hMSH2 or inactivation of other genes leading to genetic instability (15, 16). hMSH6 has been one such candidate, but genetic alteration of hMSH6 was infrequent in MSI-L CRCs (15, 19). In this study, immunohistochemical staining for hMSH6 revealed loss of expression of hMSH6 in a subset of MSI-H cancers but not in MSI-L or MSS CRC. This is similar to previous reports (15, 19). Recently, a high frequency of promoter hypermethylation of the DNA repair gene MGMT was reported in MSI-L CRCs (16). In the present series, the frequency of hypermethylation of MGMT was higher in advanced...
CRCs with MSI-L than other types, but the findings did not reach statistical significance.\(^5\)

LOH analysis of early CRCs showed that the MSI-L phenotype was significantly associated with chromosomal loss of 1p32 \((P = 0.013\) and 8p12–22 \((P = 0.005\); Table 2\). Loss of 1p32 and 8p12–22 was also observed in advanced CRCs with MSI-L. Although the biological significance of the MSI-L phenotype is uncertain \((7, 15, 16)\), the significant association with specific patterns of LOH suggests that MSI-L is unlikely to represent an unimportant epiphenomenon. Because this study was undertaken on an unselected cohort based, the number of the MSI-H samples was too small to compare with other phenotypes. Additionally, the reliability of LOH analysis in MSI-L tumors may be influenced by the hyperinstability of microsatellite markers. Thus, our analysis is limited to MSS and MSI-L phenotype in this study.

Interestingly, clinopathological analysis of advanced MSI-L CRCs revealed that MSI-L cancers were three times as likely to recur and showed a worse prognosis compared with other phenotypes, although this did not reach statistical significance.\(^5\) Our data also showed that LOH at 1p32 was an independent adverse prognostic factor,\(^5\) similar to the findings of others \((21)\). An as yet unidentified putative suppressor gene located on 1p32, therefore, appears to be associated with poor prognosis \((18)\). The cosegregation of two adverse prognostic markers with MSI-L status is interesting and requires further investigation.

There was a relationship between multiple chromosomal alterations and K-ras mutation. Early CRC cases with K-ras mutation lacked high levels of LOH on the chromosomes examined. By contrast, early CRCs with wild-type K-ras were more likely to have multiple deleterious genetic alterations, suggesting that K-ras mutation seems to be a key genetic alteration for the formation of advanced CRCs, no. (%) 116 78 (67.2) 30 (25.9) 8 (6.9)

\(\text{Table 2}. \) Summary of frequency of genetic alterations in early and advanced CRCs

<table>
<thead>
<tr>
<th>MSI status No. of tumors analyzed</th>
<th>Mutation LOH/informative tumors</th>
<th>K-ras codon 12</th>
<th>1p32</th>
<th>2p16</th>
<th>5q11-q22</th>
<th>7q31</th>
<th>8p12-p22</th>
<th>8p22</th>
<th>17p</th>
<th>17q11</th>
<th>18q21-q23</th>
</tr>
</thead>
<tbody>
<tr>
<td>Early CRCs</td>
<td>104</td>
<td>24/101 (23.8)</td>
<td>25/75 (33.3)</td>
<td>17/78 (21.8)</td>
<td>35/83 (43.2)</td>
<td>38/75 (50.7)</td>
<td>3/88 (4.4)</td>
<td>46/88 (52.3)</td>
<td>49/88 (55.7)</td>
<td>46/88 (52.3)</td>
<td>49/88 (55.7)</td>
</tr>
<tr>
<td>MSI-H</td>
<td>7 (6.7)</td>
<td>1/6 (16.7)</td>
<td>0/1 (0)</td>
<td>0/1 (0)</td>
<td>0/1 (0)</td>
<td>0/1 (0)</td>
<td>0/1 (0)</td>
<td>0/1 (0)</td>
<td>0/1 (0)</td>
<td>0/1 (0)</td>
<td>0/1 (0)</td>
</tr>
<tr>
<td>MSI-L</td>
<td>53 (51.0)</td>
<td>10/51 (19.6)</td>
<td>18/37 (48.6)</td>
<td>12/38 (31.6)</td>
<td>20/44 (45.5)</td>
<td>21/36 (58.3)</td>
<td>32/45 (71.1)</td>
<td>29/43 (67.4)</td>
<td>25/34 (73.5)</td>
<td>8/20 (40.0)</td>
<td>23/36 (63.9)</td>
</tr>
<tr>
<td>MSS</td>
<td>44 (42.3)</td>
<td>13/44 (29.5)</td>
<td>7/37 (18.9)</td>
<td>5/39 (12.8)</td>
<td>15/39 (38.5)</td>
<td>17/39 (43.6)</td>
<td>16/39 (41.0)</td>
<td>19/39 (49.5)</td>
<td>9/30 (30.0)</td>
<td>22/37 (59.5)</td>
<td>9/30 (30.0)</td>
</tr>
<tr>
<td>Advanced CRCs</td>
<td>116</td>
<td>27/114 (23.7)</td>
<td>15/86 (17.4)</td>
<td>10/78 (12.8)</td>
<td>28/91 (30.8)</td>
<td>35/92 (38.0)</td>
<td>42/103 (40.8)</td>
<td>37/96 (38.5)</td>
<td>30/73 (41.1)</td>
<td>16/68 (23.5)</td>
<td>42/91 (46.2)</td>
</tr>
<tr>
<td>MSI-H</td>
<td>8 (6.9)</td>
<td>1/8 (12.5)</td>
<td>0/1 (0)</td>
<td>—</td>
<td>1/1 (100.0)</td>
<td>1/3 (33.3)</td>
<td>4/6 (66.7)</td>
<td>4/6 (66.7)</td>
<td>2/3 (66.7)</td>
<td>0/1 (0)</td>
<td>2/4 (50.0)</td>
</tr>
<tr>
<td>MSI-L</td>
<td>30 (25.9)</td>
<td>12/29 (41.4)</td>
<td>6/15 (40.0)</td>
<td>7/18 (38.9)</td>
<td>10/21 (47.6)</td>
<td>15/22 (68.2)</td>
<td>15/25 (60.0)</td>
<td>14/23 (60.9)</td>
<td>11/18 (61.1)</td>
<td>8/16 (50.0)</td>
<td>10/22 (45.5)</td>
</tr>
<tr>
<td>MSS</td>
<td>78 (67.2)</td>
<td>14/77 (18.2)</td>
<td>9/70 (12.9)</td>
<td>3/60 (5.0)</td>
<td>17/69 (24.6)</td>
<td>19/67 (28.4)</td>
<td>23/72 (31.9)</td>
<td>19/67 (28.4)</td>
<td>17/52 (32.7)</td>
<td>8/43 (15.7)</td>
<td>30/65 (46.2)</td>
</tr>
</tbody>
</table>

\(P^a\) Fisher's exact test (MSI-L versus MSS). \(P^a\) was two-tailed and statistically significant when \(P < 0.05\).
early CRCs. In advanced CRCs, \textit{K-ras} mutation showed no relationship with LOH. With respect to MSI-L status, \textit{K-ras} mutation was more frequently observed in advanced MSI-L CRCs ($P = 0.022$), whereas no such correlation was observed in early CRCs with MSI-L (Table 2).

Comparison of MSI-L frequency in early and advanced CRCs raises several questions, particularly with respect to the lower frequency of MSI-L cancer in advanced versus early cancer. It is known that the evolution of cancer requires the continued development of subclones, and that only subclones with a growth advantage will progress (23). It is possible that subclones comprising MSI-L cancer cells are less likely to develop to advanced cancer, and that small MSS clones ultimately prevail as a result of clonal selection. Conceivably, a subclone of MSI-L CRC with wild-type \textit{K-ras} and characterized by multiple loci showing LOH may not be fully resistant to further DNA damage and may therefore succumb through apoptotic deletion. Acquisition of \textit{K-ras} mutation could be the factor that triggers this outcome in view of the negative correlation between \textit{K-ras} mutation and extensive LOH in early CRCs. The high frequency of \textit{K-ras} mutation observed in advanced MSI-L CRCs but not in early MSI-L CRCs may also support this idea. Another possible explanation is transient hypermethylation (with subsequent demethylation) of genes, e.g., \textit{hMLH1} or \textit{MGMT}, implicated directly or indirectly in the development of MSI. Nevertheless, it is likely that early MSI-L cancers give rise to advanced MSI-L cancers in light of the frequent findings of 1p and 8p LOH in both groups.

Regardless of these speculations, we were able to demonstrate that MSI-L is a frequent genetic event in early CRCs with invasion limited to the submucosa. Putative tumor suppressor genes located at 1p and 8p may be involved in MSI-L carcinogenesis. Further research is needed to clarify the mechanisms driving the MSI-L phenotype and whether these are similar in Japan and the west.

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