Colon cancers displaying microsatellite instability (MSI) are clinically less aggressive. Based on in vitro studies and recent clinical data, cancers displaying MSI do not respond to 5-fluorouracil (5-FU). The reasons why MSI tumors are clinically less aggressive and do not respond to 5-FU-based therapies have not been fully elucidated.

**Purpose:** We investigated biomolecular markers in an attempt to explain the different clinical behavior and chemotherapeutic responses of MSI and non-MSI colon cancers.

**Experimental Design:** One hundred ninety-two sporadic colon cancers were tested for MSI with five mononucleotide markers and methylation of the hMLH1 promoter. Slides were stained for thymidylate synthase (TS), p53, MDM2, p21^{WAF1/CIP1}, β-catenin, vascular endothelial growth factor, hMLH1, hMSH2, and hMSH6. Tumors were regarded as having wild-type, functional p53 (Fp53) if reduced expression of p53 and positive MDM2 and p21^{WAF1/CIP1} expressions were found.

**Results:** Of the cases, 12.5% were MSI-H (at least two markers mutated). Of MSI-H cases, 83.3% were characterized by a complete loss of at least one of the mismatch repair proteins, in particular loss of hMLH1 by promoter hypermethylation. MSI-H colon cancers showed higher expression of TS compared with MSS (no mutated markers)/MSI-L (one mutated marker) colon cancers (66.6% for MSI-H versus 14.8% MSS/MSI-L; \( P < 0.0001 \)); 20.8% of MSI-H cases showed high expression of the vascular endothelial growth factor, compared with 45.8% MSS/MSI-L colon cancers (\( P = 0.0005 \)); 45.8% MSI-H cases had Fp53 compared 11.9% MSS/MSI-L cases (\( P < 0.0001 \)).

**Conclusions:** About 12% of colon cancers display MSI mostly due to lack of hMLH1 resulting from promoter hypermethylation. These tumors have high expression of TS and retain fully functional p53 system. Thus, these data suggest why sporadic hMLH1-defective colon cancers often do not respond to 5-FU.

Colorectal cancer is among the most common forms of diagnosed neoplasias and the second leading cause of cancer death worldwide. Five-year survival rates for surgically resected colon cancer remains low despite several efforts having been made to target fluorouracil-based chemotherapies.
fragment analysis used with a Beckman Coulter sequencer CEQ 2000 XL (Beckman Coulter S.p.A., Milan, Italy) as previously described (37). For statistical purposes, MSI-L (one mutated marker) cancers were considered together with MSS (no mutated markers; ref. 36).

**Immunohistochemistry and image cytometry.** Serial sections of formalin-fixed, paraffin-embedded samples were stained according to an amplified nonbiotin peroxidase system. Sections were dewaxed, rehydrated, and subjected to antigen retrieval treatment. Sections stained using monoclonal anti-p53 (clone BP53.12, Novocastra Laboratories, Newcastle-upon-Tyne, England), anti-β-catenin (clone 17C2, Novocastra Laboratories), anti hMLH1 (clone G166-15, Pharmingen, San Diego, CA), anti hMSH6 (clone 44, Transduction Lab, San Diego, CA), and polyclonal anti–vascular endothelial growth factor (VEGF; Zymed Laboratories, Inc., South San Francisco, CA) antibodies were treated with citrate buffer (pH 6.0) at 98°C for 40 minutes.

Tissues incubated with monoclonal anti-p21WAF1/CIP1 (clone EA10, Oncogene Research Products, San Diego, CA), anti hMSH2 (clone FE11, Zymed Laboratories), and anti-TS (clone TS106, Zymed Laboratories) antibodies were treated with EDTA buffer (pH 8.0) in a microwave oven for 10 minutes at 750 W. After cooling at room temperature, endogenous peroxidase activity was inhibited using a methanol/H2O2 solution (0.5%) for 20 minutes. Sections were then washed in PBS (pH 7.2-7.4) and processed using SS-HPR nonbiotin system according to the manufacturer-suggested procedure (BioGenex Lab., San Ramon, CA). Incubation was done overnight at room temperature in a humidified atmosphere. The immunologic reaction was developed using a 3,3-diaminobenzidine/H2O2 PBS solution.

Immune serum was omitted in negative controls. Tumor sections previously determined as positive for p53, p21WAF1/CIP1, VEGF and hMLH1, hMSH2, and hMSH6 expression were used as positive control. Sections from formalin-fixed, paraffin-embedded SW480 cell line previously assayed for TS expression (38) were run in each batch of TS slides as positive control. If present, normal tissue adjacent to neoplasia was considered as an internal control (TS expression in basal cells; hMLH1/hMSH2/hMSH6 expression in normal cell nuclei). Scattered p53 as well as hMLH1/hMSH2/hMSH6-positive nuclei were also present through the stromal compartment of neoplasia to confirm complete loss of expression for these proteins.

**Scoring of immunohistochemical staining.** Cytoplasmic TS and VEGF immunoreactive population was evaluated according to positive tumor cell percentage and staining intensity (39) as follows: score 0 if <1%, score 1 if >1% <20%, score 2 if >20% <50%, score 3 if >50% <80%, score 4 if >80%; intensity: score 1 (weak), score 2 (moderate), and score 3 (strong). Where the neoplastic population showed a patchy, nonuniform staining intensity, the value was referred to the prevalent immunostained intensity. A final classification was obtained by combining the two score values (sum) as follows: negative (sum range 0-2), low (3-5), and high (6-7) expression, respectively. Nuclear immunostaining for p53, p21WAF1/CIP1, β-catenin (Nu-β-cat), MDM2, hMLH1, hMSH2, and hMSH6 was quantified by image cytometry with Cytometrica software (C&V, Bologna, Italy) as previously detailed (40). A labeling index was obtained and expressed as the percentage of the labeled nuclear area over the total neoplastic nuclear area (%)La. Nuclear immunostaining was classified using the following cutoff values: p53 and p21 negative if <10% La; positive if >10% La (41, 42). β-catenin <10% = negative, >10% = positive. hMLH1, hMSH2, and hMSH6 expressions were considered negative if <1% and positive if >1%. MDM2 nuclear expression was arbitrarily classified using the mean %La value (2.2% La): negative if <2.2% La; positive if >2.2% La. The concomitant findings of p53 <10% La, MDM2 >2.2% La, and p21WAF1/CIP1 >10% La were considered suggestive for a functional p53 system (Fp53).

To the best of our knowledge, nuclear TS expression was not previously evaluated; in this view, we arbitrarily chose the following cutoff values: nuTS negative <10% La; nuTS low >10% <30% La; and nuTS high >30% La. Because TS clone TS106 antibody recognizes both nuclear and cytoplasmic TS (NeoMarkers Lab clone TS106 information...
Categorical variables were tested using Fisher’s exact test and visualized after ethidium bromide staining. PCR products were run in a 4% Nu-sieve GTG agarose gel in Tris-borate- and bisulfite-treated DNA extracted from the cell line RKO were analyzed as previously described (43) after modifying PCR conditions.

region C of the hMLH1 promoter, which correlates with loss of protein expression as previously described (43) after modifying PCR conditions. The method of analysis and the test for statistical significance depended on the nature of the concerned variables. Site distribution of mean continuous values was tested using unpaired t test. Statistical analysis. The method of analysis and the test for statistical significance depended on the nature of the concerned variables. Site distribution of mean continuous values was tested using unpaired t test. Factor analysis using principal component method and correspondence analysis were used in our study to test possible associations between several continuous variables in a single analysis (principal component) or to graphically plot categories disclosing similarities or associations among variables. Correspondence analysis permits graphical highlighting of the associations in two-way and multiway contingency tables. Correspondence analysis focuses primarily on data reduction and interpretation and generates graphical scatter plots where different categories are displayed as points. The relative positions of the points indicate different degrees of similarity or association among categories. The applications of correspondence analysis techniques in the medical research fields are essentially exploratory in nature and are extensively explained in existing literature (44). Correspondence analysis was done with the BMDP statistical software (Los Angeles, CA). All other analyses were done with StatView 5.0 software (SAS Institute, Cary, NC).

Results

Sample locations and immunohistochemical features. We analyzed 192 consecutive sporadic colon cancer samples. Eighty-three (43.2%) cases were located in the right colon and 109 (56.8%) in the left colon. Pathologic grading (WHO criteria) disclosed 21 (10.9%) well differentiated, 143 (74.5%) moderately differentiated, and 28 (14.6%) poorly differentiated cases, respectively. We found an increase in poorly differentiated tumors in the right colon (19 of 83 right colon versus 9 of 109 left colon), whereas well-differentiated tumors were more frequent in the left colon (8 of 83 right colon versus 13 of 109 left colon; P = 0.02). Twenty-nine of 192 (15.1%) cases were mucinous (>50% of the area composed by extracellular mucin) with a prevalent, but not significant, distribution in the right colon, 16 of 83 (19.3%), versus left colon, 13 of 109 (11.9%). According to pTNM (Unio Internationale Contra Cancrum 5th edition) criteria, 90 cases were stage II (46.9%), 77 stage III (40.1%), and 25 stage IV (13.0%), respectively. No significant association between anatomic distribution and staging was found.

Cancers resulted to be more negative for hMLH1 protein expression in the right colon compared with the left colon. On the other hand, p21/WAF1/CIP1 and MDM2 were more expressed in the left colon. Similar expressions of TS, hMSH2, hMSH6, and p53 were found in right and left colon cancers (see Table 1).

Factor analysis conducted on the continuous variables using the principal component method showed three significant (>1.0 of magnitude) associations. The first factor (eigenvalue = 2.005) showed an inverse relationship between hMLH1 factor loading (FL) = −0.735 and TS (FL = 0.872) expression. The second factor (eigenvalue = 1.671) revealed a relationship between hMSH2 and hMSH6 (FL = 0.782 and 0.822, respectively). Finally, the third association (eigenvalue = 1.002) showed a relationship between MDM2 and p21/WAF1/CIP1 (FL = 0.687 and 0.570, respectively), both inversely related to p53 expression (FL = −0.858).

Microsatellite instability status compared with hMLH1 promoter methylation and mismatch repair protein expressions. We determined MSI by testing colon cancer cases with five mononucleotide markers (Fig. 1A) and classified accordingly. We found 24 cases (12.5%) as MSI-H, 17 (8.9%) as MSI-L, and 151 (78.6%) as MSS. Twenty of 24 MSI-H cases (83.3%) were characterized by a complete loss of at least one of the mismatch repair proteins considered. Nineteen of 20 MSI-H cases revealed a relationship between MDM2 and p21/WAF1/CIP1 (FL = 0.687 and 0.570, respectively), both inversely related to p53 expression (FL = −0.858).

Table 1. Distribution of biopathologic parameters in the right and left colon

<table>
<thead>
<tr>
<th></th>
<th>Right-located CRCs</th>
<th>Left-located CRCs</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>MLH1</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Neg</td>
<td>18</td>
<td>2</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>Pos</td>
<td>65</td>
<td>107</td>
<td></td>
</tr>
<tr>
<td>MSH2</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Neg</td>
<td>5</td>
<td>4</td>
<td>= n.s.</td>
</tr>
<tr>
<td>Pos</td>
<td>78</td>
<td>105</td>
<td></td>
</tr>
<tr>
<td>MSH6</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Neg</td>
<td>2</td>
<td>2</td>
<td>= n.s.</td>
</tr>
<tr>
<td>Pos</td>
<td>81</td>
<td>107</td>
<td></td>
</tr>
<tr>
<td>p53</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Neg</td>
<td>31</td>
<td>38</td>
<td>= n.s.</td>
</tr>
<tr>
<td>Pos</td>
<td>52</td>
<td>71</td>
<td></td>
</tr>
<tr>
<td>p21</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Neg</td>
<td>25</td>
<td>61</td>
<td>&lt;0.0004</td>
</tr>
<tr>
<td>Pos</td>
<td>58</td>
<td>48</td>
<td></td>
</tr>
<tr>
<td>MDM2</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Neg</td>
<td>45</td>
<td>83</td>
<td>= 0.0014</td>
</tr>
<tr>
<td>Pos</td>
<td>38</td>
<td>26</td>
<td></td>
</tr>
<tr>
<td>nu3-cat</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Neg</td>
<td>46</td>
<td>33</td>
<td>&lt;0.0005</td>
</tr>
<tr>
<td>Pos</td>
<td>37</td>
<td>76</td>
<td></td>
</tr>
<tr>
<td>TS</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Neg</td>
<td>33</td>
<td>55</td>
<td>= n.s.</td>
</tr>
<tr>
<td>Low</td>
<td>30</td>
<td>33</td>
<td></td>
</tr>
<tr>
<td>High</td>
<td>20</td>
<td>21</td>
<td></td>
</tr>
<tr>
<td>VEGF</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Neg</td>
<td>20</td>
<td>9</td>
<td>= 0.003</td>
</tr>
<tr>
<td>Low</td>
<td>35</td>
<td>44</td>
<td></td>
</tr>
<tr>
<td>High</td>
<td>27</td>
<td>56</td>
<td></td>
</tr>
<tr>
<td>MSI</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>MSI-H</td>
<td>18</td>
<td>6</td>
<td>&lt;0.0008</td>
</tr>
<tr>
<td>MSS/MSI-L</td>
<td>6</td>
<td>103</td>
<td></td>
</tr>
</tbody>
</table>

Abbreviations: CRC, colon cancer; n.s., not significant; Neg, negative; Pos, positive.
Of the MSI-H colon cancers, 18 (75%) were located in the right colon whereas 6 (25%) were in the left. This prevalent right site location was statistically significant (P = 0.0008). Using Fisher’s exact t test for MSI status and mismatch repair protein evaluation, we found a significant difference between MSI-H and MSS/MSI-L cases for hMLH1 and hMSH6 expressions. No statistical significance was found for hMSH2 expression (see Table 2).

**Microsatellite instability status and pathologic parameters.** Mean age for MSI status revealed no significant difference (Table 2). MSI-H cases showed more poorly differentiated and less well-differentiated carcinomas than MSS/MSI-L cases (P < 0.0001). Furthermore, MSI-H cases showed a higher mucinous histotype compared with MSS/MSI-L colon cancers (P = 0.04). No significant association was found between MSI and pathologic stage.

**Microsatellite instability status, thymidylate synthase, p53 system, vascular endothelial growth factor, and β-catenin expression.** As shown in Table 2 and Fig. 2, we found that MSI-H cases had a strikingly higher expression of TS compared with MSS/MSI-L (16 of 24, 66.6%, for MSI-H; 25 of 168, 14.8%, for MSS/MSI-L; P < 0.0001). Furthermore, MSI-H cases showed a significant reduction of VEGF expression and p53 nuclear accumulation. No differences were found for Nuβ-cat among the tested groups. On the contrary, they displayed a high expression of both p21^{WAF1/CIP1} and MDM2. We have considered a significant coexpression of MDM2 (>2.2% LIa) and p21^{WAF1/CIP1} (>10% LIa) proteins together with a low (<10% LIa) p53 accumulation as suggestive for “active” wild-type p53 system. Cases were classified as p53 nonfunctioning [NFp53 (p53 ≥ 10% LIa, MDM2 <2.2% LIa, and p21^{WAF1/CIP1} < 10% LIa)] or functioning [Fp53 (p53 < 10% LIa, MDM2 ≥ 2.2% LIa, and p21^{WAF1/CIP1} ≥ 10% LIa)]. MSI-H cases showed a highly significant association with Fp53 compared with MSS/MSI-L (P < 0.0001). We also found 29 cases with complete loss of p53 nuclear immunostaining (0%), but all these cases showed no MDM2 expression (from 0% to 0.9% LIa) and were classified as NFp53 cases. Correspondence analysis allowed us to define the cluster association between the different analysis considered. In the bidimensional CA map (Fig. 3), the relative position of MSI-H tumors was broadly associated to TS high (TS-H), functional p53 (Fp53), and VEGF-negative (VEGF-N) categories (as shown in the box), suggesting a strong statistical association between them. On the other hand, MSS cancers were strongly associated to nonfunctional p53 (NFp53) and high expression of VEGF (VEGF-H).

**Discussion**

In the present study, we confirm that 12% of all colorectal cancers display MSI, mostly due to hMLH1 promoter

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**Fig. 1.** A, sample 45 displays variations at both BAT25 and BAT26 markers, whereas sample 128 is stable for both. B, methylation-specific PCR for the hMLH1 promoter. Samples 2, 4, 45, and 107 display both the unmethylated (U) and the methylated (M) bands, confirming methylation of the promoter. On the other hand, samples 121, 124, and 141 display just the unmethylated fragment. MW, molecular weight. B, blank.
hypermethylation (11, 12, 45) and that the majority of these tumors are right-side located and mucinous. More importantly, our study shows, for the first time, that cancers with MSI and defective mismatch repair system have high expression of TS, which is a key factor for 5-FU response as shown by several studies showing that cancers with high levels of TS have a poor response to 5-FU, whereas those displaying low levels of TS do respond to the chemotherapeutic agent (28, 29, 46). Furthermore, patients with polymorphism of the enhancer region of the TS promoter, which is associated with higher TS levels, have a low survival benefit from standard 5-FU chemotherapy (47).

For statistical purposes, we combined samples obtained from the rectum with those from the sigmoid and descending colon after finding no differences between them for all the analyzed parameters (data not shown). Because we did not have cancer and normal-paired tissues, we used five mononucleotide repeat markers that were shown in a previous study to be quasimonomorphic in normal DNA and to be effective markers for determining the MSI status of human tumors (48). Our results found concordance between MSI-H and loss of mismatch repair protein expression, in particular hMLH1, verified by immunohistochemistry.

Some of previous reports used BAT26 alone to define MSI cancers. Elsaleh et al. (49, 50), on the basis of the BAT26 marker, suggested that MSI cancers are sensitive to 5-FU–based chemotherapy. Others also confirmed that patients with MSI cancers had good prognosis from 5-FU–based treatments (51, 52). However, recent data obtained with the use of more restricted criteria for the definition of MSI found that these type cancers were less sensitive to 5-FU chemotherapy than patients with more extensive MSI.

### Table 2. Evaluation of MSI status, pathologic parameters, and mismatch repair protein expression

<table>
<thead>
<tr>
<th></th>
<th>MSHL (24 cases)</th>
<th>MSS (168 cases)</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (mean ± SE)</td>
<td>71.2 ± 2.3 SE</td>
<td>66.8 ± 1.0 SE</td>
<td>n.s.</td>
</tr>
<tr>
<td>Grade [Well (W), Moderate (M), Poor (P)]</td>
<td>W 2, M 10, P12</td>
<td>W 19, M 133, P15</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>Mucinous istotype (Yes, No)</td>
<td>Yes 7, No 17</td>
<td>Yes 22, No 146</td>
<td>0.04</td>
</tr>
<tr>
<td>Stage (Stg II, III, IV)</td>
<td>Stg II 12, Stg III 10, Stg IV 2</td>
<td>Stg II 78, Stg III 67, Stg IV 23</td>
<td>n.s.</td>
</tr>
<tr>
<td>hMLH1 %Lia (mean ± SE)</td>
<td>16.8 ± 7.4</td>
<td>81.1 ± 1.5</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>hMSH2 %Lia (mean ± SE)</td>
<td>49.1 ± 6.2</td>
<td>59.3 ± 2.2</td>
<td>n.s.</td>
</tr>
<tr>
<td>hMSH6 %Lia (mean ± SE)</td>
<td>53.6 ± 5.6</td>
<td>69.2 ± 1.8</td>
<td>0.003</td>
</tr>
<tr>
<td>p53 (Neg vs Pos)</td>
<td>13 Neg, 11 Pos</td>
<td>56 Neg, 112 Pos</td>
<td>0.05</td>
</tr>
<tr>
<td>p21 (Neg vs Pos)</td>
<td>4 Neg, 20 Pos</td>
<td>82 Neg, 86 Pos</td>
<td>0.003</td>
</tr>
<tr>
<td>MDM-2 (Neg vs Pos)</td>
<td>8 Neg, 16 Pos</td>
<td>114 Neg, 54 Pos</td>
<td>0.001</td>
</tr>
<tr>
<td>nu-cat (Neg vs Pos)</td>
<td>14 Neg, 10 Pos</td>
<td>65 Neg, 103 Pos</td>
<td>n.s.</td>
</tr>
<tr>
<td>VEGF (Neg vs low vs High)</td>
<td>10 Neg, 9 Low, 5 High</td>
<td>20 Neg, 71 Low, 77 High</td>
<td>0.0005</td>
</tr>
<tr>
<td>TS (Neg vs Low vs High)</td>
<td>1 Neg, 7 Low, 16 High</td>
<td>87 Neg, 56 Low, 25 High</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>Fp53 (NFp53 vs Fp53)</td>
<td>11 NFp53, 13 Fp53</td>
<td>148 NFp53, 20 Fp53</td>
<td>&lt;0.0001</td>
</tr>
</tbody>
</table>

Fig. 2. Immunohistochemistry of a sample with MSI-H due to hypermethylation of the hMLH1 promoter, which resulted negative for hMLH1 protein expression, with reduced expression of p53, negative for VEGF (not shown in picture), and with high expression of MDM2, p21, TS.
of cancers do not respond to 5-FU–based chemotherapy, which is in accordance to in vitro data (53, 54).

Thus, the discrepancy in the studies might be associated to the lack of consistency in the chosen microsatellite markers to define MSI cancers. Interestingly, one of the previously cited studies verified if the MSI phenotype was associated to the loss of one of the mismatch repair proteins at least by immunohistochemistry.

We also found that MSI-H cancers retain a fully functional p53 system and confirmed that they have lower VEGF expression than non-MSI cancers (55). We defined the p53 system as functional by the association of low expression of p53 and high expression of the MDM2 cofactor, and p21WAF1/CIP1, although p21WAF1/CIP1 can also accumulate in a p53-independent pathway related to cell cycle arrest due to senescence, terminal differentiation, and apoptosis (56).

We also found complete loss of p53 and MDM2 immunostaining in 29 cases indicating an underlying p53 mutation as previously suggested (57), that were considered as nonfunctioning. p53 expression has been used as a tool to indicate cancers that might or might not be sensitive to 5-FU–based chemotherapy (49). Furthermore, p53 has been used as a prognostic marker irrespective of chemotherapy, with conflicting results (58–62). It has also been suggested that patients with stage III colon cancer with wild-type Ki-ras or no p53 expression would benefit from adjuvant 5-FU plus levamisole (63); however, the authors did not discriminate between MSI and non-MSI cancers. We believe that the retained p53 function of MSI cancers might explain why these tumors have a better prognosis compared with non-MSI cancers. Moreover, MSI cancers seem to be less aggressive (14), irrespective with the 5-FU regimens, as also suggested by the lack of VEGF expression found in our series, which is in accordance with two independent groups who defined that among subjects who did not receive 5-FU, those which is in accordance with two independent groups who suggested by the lack of VEGF expression found in our series, that eventually would not respond to the 5-FU–based chemotherapy (49).

Our data give support to previous reports and indicate that MSI cancers mostly due to hMLH1 promoter hypermethylation would not respond to 5-FU relating this phenomenon to the high expression of TS of these tumors. Finally, we suggest the use of multiple mononucleotide MSI markers to detect cancers that eventually would not respond to the 5-FU–based regimens.

Acknowledgments

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