High Thymidylate Synthase Expression in Colorectal Cancer with Microsatellite Instability: Implications for Chemotherapeutic Strategies

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Abstract 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.

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In recent years, several milestones have been achieved for the comprehension of the genetic basis of the disease (1). Almost 85% of all colon cancers display chromosomal instability, which is characterized by loss of heterozygosity, gross chromosomal rearrangements, and aneuploidy (2, 3). On the other hand, the failure of the human DNA mismatch repair system leads to the development of the so-called mutator phenotype, which is characterized by increased replication errors at highly repetitive short DNA sequences, termed microsatellite instability (MSI; refs. 4–8). Essentially 100% of sporadic MSI colorectal cancers are due to hMLH1 promoter hypermethylation (9–12). Cancers showing MSI are typically diploid, metastasize infrequently, and, although pathologically with higher grade, have a better prognosis than chromosomal instability tumors (13, 14). Cancers with chromosomal instability usually display increased mutated p53 levels. p53 regulates proper cell cycle, apoptosis, and angiogenesis (15–17). In particular, p53 has an antiangiogenic activity by inducing the expression of the *Maspin* gene that has been shown to prevent cell invasion and metastasis in breast cancer (18). Wild-type p53 protein is regulated by a feedback loop: p53 induces MDM2, which in turn targets p53 promoting its

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degradation (19, 20). Phosphorylation of wild-type p53 induced by DNA damage prevents the interaction between p53 and MDM2, thus abrogating the protein degradation leading to the accumulation of p53 and p53-responsive proteins (21, 22). p21$^{WAF1/CIP1}$, a cyclin inhibitor kinase, is a downstream target of the p53 loop, important for the inhibition of cell cycle after DNA damage and consequent repair (23 – 25). MSI cancers usually display normal levels of p53 although the function of the entire p53 system has not been fully evaluated in MSI cancers in previous studies.

5-Fluorouracil (5-FU) is the chemotherapeutic agent of first choice for treatment of colon cancer in combination with leucovorin in the adjuvant setting (26) or with either Irinotecan or oxaliplatin for metastatic disease (27). The thymidylate synthase (TS) enzyme catalyzes conversion of dUMP to dTMP, the latter being necessary for DNA synthesis. Inhibition of TS activity is thought to be one of the major mechanisms for the anticancer effects of 5-FU. The cytotoxic effect of this drug occurs through the action of FdUMP, a 5-FU metabolite competing with dUMP for binding the TS protein. In several studies of tumor cell lines and clinical samples, the TS activity level was found to be predictive for response to 5-FU. Most, but not all (28 – 31), studies report that high tumor TS levels are associated with poor response to 5-FU – based chemotherapy. A recent study found that advanced colon cancer patients with low tumor levels of TS showed much greater survival benefit from 5-FU than those with high expression levels (32). Furthermore, *in vitro* work with 5-FU indicated that colon cancer cells with MSI hardly respond to this agent, whereas cells proficient in mismatch repair do not survive to 5-FU treatment compared with MSI colon cancer cells (33). Finally, a cell line displaying MSI due to hypermethylation of the hMLH1 promoter, which is resistant to 5-FU, becomes sensitive after the re-expression of hMLH1 after demethylation with 5-azadeoxycytidine (34).

In the present study, we wanted to fully characterize consecutive human colon cancers for several biological and molecular markers to explain clinical and chemotherapeutic behaviors of MSI and non MSI colon cancers. The results may be useful in providing predictive or prognostic information before starting chemotherapy and to define patients' survival.

Materials and Methods

Tissue samples and DNA extraction. We obtained formalin-fixed, paraffin-embedded tissue samples from 192 consecutive patients surgically resected for sporadic colorectal cancer (i.e., not belonging to hereditary nonpolyposis colon cancer or familial adenomatous polyposis families) during the year 2001 at Policlinico S.Orsola-Malpighi, Bologna, Italy. Eighty-three cases were located in the right colon (up to the splenic flexure) and 109 in the left colon (descending, sigmoid and rectum). Age and gender distribution was as follows: 32 to 92 years (67.3 ± 12.1, mean ± SD); 107 male and 85 female. For pathologic grading, we used the WHO criteria. Staging was done according to pTNM [Unio Internationale Contra Cancrum 5th edition] criteria. DNA extraction was done by tissue microdissection and DNA isolation was done with the QIAmp DNA extraction kit (Qiagen, S.p.A., Milan, Italy) following the manufacturer's suggestions.

Microsatellite instability testing. Because normal tissue was not available for all samples, we assessed MSI by analyzing five mononucleotide markers as previously described (35) and suggested by the National Cancer Institute workshop (36), and MSI cancers were classified accordingly. MSI analysis was done by fluorescent PCR followed by 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-MDM2 (clone SMP14, Neo-Markers, Freemont, CA), anti – β-catenin (clone 17C2, Novocastra Laboratories), anti hMLH1 (clone G168-15, 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-p21$^{WAF1/CIP1}$ (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/H_2O_2 solution (0.5%) for 20 minutes. Sections were than washed in PBS (pH 7.2-7.4) and processed using SS-HRP 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/H_2O_2 PBS solution.

Immune serum was omitted in negative controls. Tumor sections previously determined as positive for p53, p21$^{WAF1/CIP1}$, 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, p21$^{WAF1/CIP1}$, β-catenin (Nuβ-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 (%LIa). Nuclear immunostaining was classified using the following cutoff values: p53 and p21 negative if <10% LIa; positive if >10% LIa (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 %LIa value (2.2% LIa): negative if <2.2% LIa; positive if >2.2% LIa. The concomitant findings of p53 < 10% LIa, MDM2 >2.2% LIa, and p21$^{WAF1/CIP1}$ >10% LIa 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% LIa; nuTS low >10% <30% LIa; nuTS high >30% LIa. Because TS clone TS106 antibody recognizes both nuclear and cytoplasmic TS (NeoMarkers Lab clone TS106 information

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

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

Abbreviations: CRC, colon cancer; n.s., not significant; Neg, negative; Pos, positive.

sheet and literature), the two informations, obtained as detailed above, were then combined together as follows: TS negative if both the cytoplasmic and the nuclear scores were negative; TS high if both scores were high; TS low if otherwise.

Methylation-specific PCR. DNA samples were bisulfite treated following published protocols and methylation-specific PCR was done for the region C of the hMLH1 promoter, which correlates with loss of protein expression as previously described (43) after modifying PCR conditions. Water- and bisulfite-treated DNA extracted from the cell line RKO were used as negative and positive controls for each reaction. Ten microliters of PCR product were run in a 4% Nu-sieve GTG agarose gel in Tris-borate EDTA buffer and visualized after ethidium bromide staining.

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. Categorical variables were tested using Fisher's exact *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 right colon compared with Nuβ-Cat and VEGF that 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 were due to loss of hMLH1 protein expression (95%). hMLH1 promoter hypermethylation was found in 16 of 19 hMLH1 defective cancers (84.2%; Fig. 1B).

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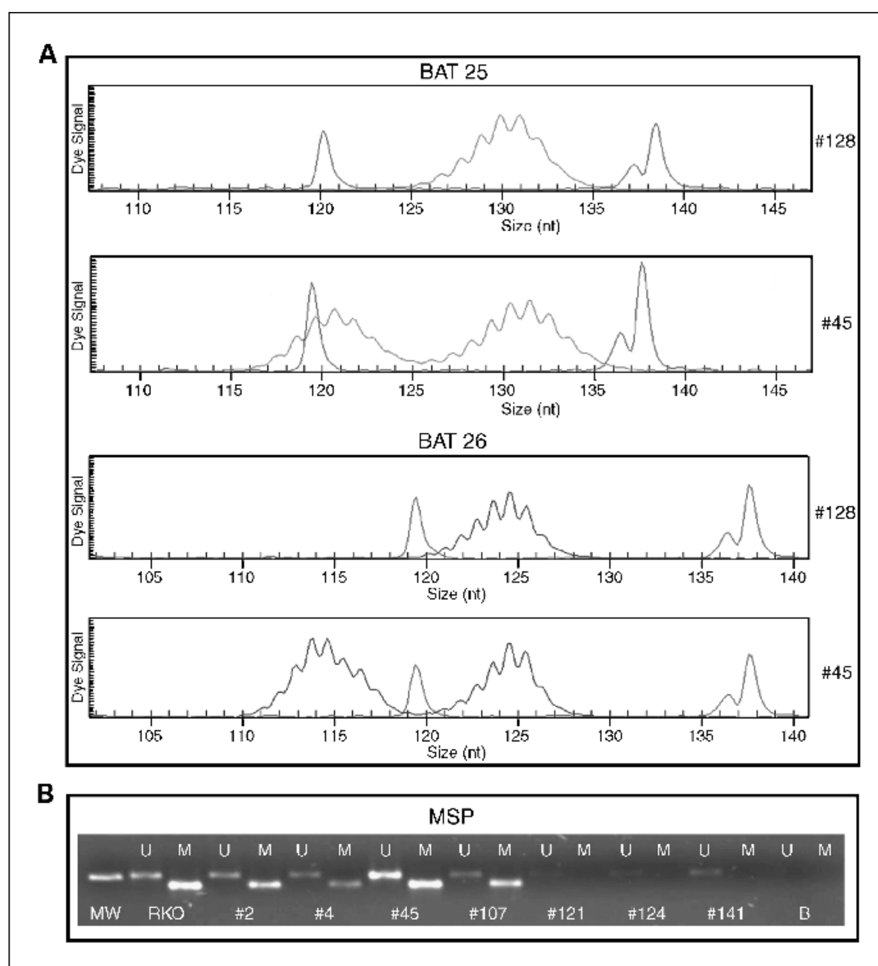

Fig. 1. *A*, sample 45 displays variations at both BAT25 and BAT26 markers, whereas sample 128 is stable for both. *B*, methylation-specific PCR for the hMLH1 promoter. Samples 2, 4, 45, and 107 display both the unmethylated (*U*) and the methylated (*M*) bands, confirming methylation of the promoter. On the other hand, samples 121, 124, and 141 display just the unmethylated fragment. *MW*, molecular weight. *B*, blank.

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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

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

	MSI.H (24 cases)	MSS (168 cases)	*P*
Age (mean±SE)	71.2 ± 2.3 SE	66.8 ± 1.0 SE	n.s.
Grade [Well (W), Moderate (M), Poor (P)]	W 2, M 10, P 12	W 19, M 133, P 15	<0.0001
Mucinous istotype (Yes, No)	Yes 7, No 17	Yes 22, No 146	0.04
Stage (Stg II, III, IV)	Stg II 12, Stg III 10, Stg IV 2	Stg II 78, Stg III 67, Stg IV 23	n.s.
hMLH1 %LIa (mean±SE)	16.8 ± 7.4	81.1 ± 1.5	<0.0001
hMSH2 %LIa (mean±SE)	49.1 ± 6.2	59.3 ± 2.2	n.s.
hMSH6 %LIa (mean±SE)	53.6 ± 5.6	69.2 ± 1.8	0.003
p53 (Neg vs Pos)	13 Neg, 11 Pos	56 Neg, 112 Pos	0.05
p21 (Neg vs Pos)	4 Neg, 20 Pos	82 Neg, 86 Pos	0.003
MDM-2 (Neg vs Pos)	8 Neg, 16 Pos	114 Neg, 54 Pos	0.001
nuβ-cat (Neg vs Pos)	14 Neg, 10 Pos	65 Neg, 103 Pos	n.s.
VEGF (Neg vs low vs High)	10 Neg, 9 Low, 5 High	20 Neg, 71 Low, 77 High	0.0005
TS (Neg vs Low vs High)	1 Neg, 7 Low, 16 High	87 Neg, 56 Low, 25 High	<0.0001
Fp53 (NFp53 vs Fp53)	11 NFp53, 13 Fp53	148 NFp53, 20 Fp53	<0.0001

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

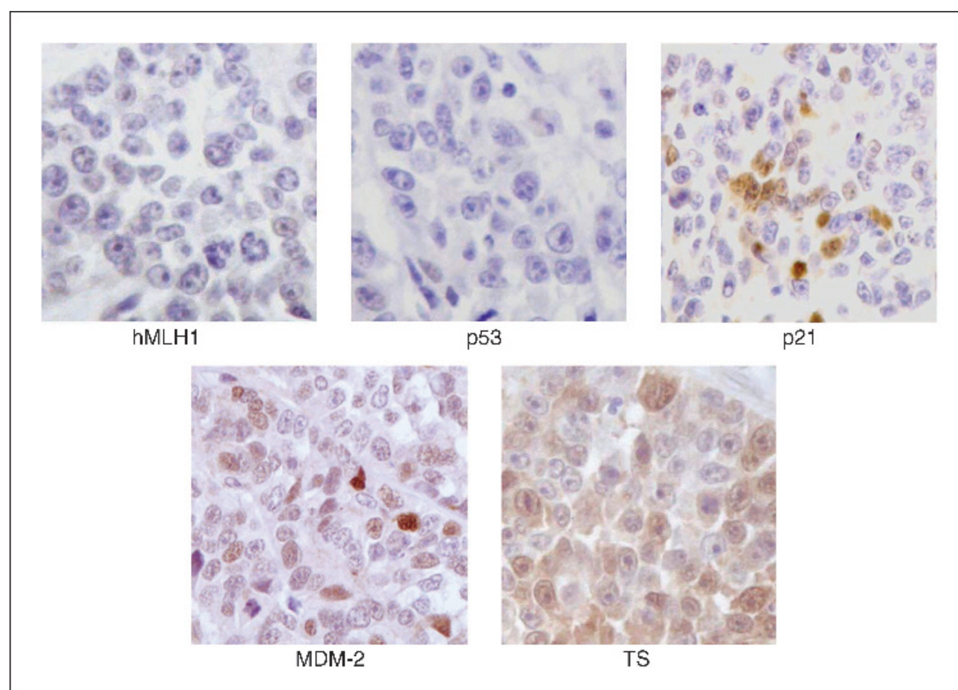

Fig. 2. Immunohistochemistry of a sample with MSI-H due to hypermethylation of the hMLH1 promoter, which resulted negative for hMLH1 protein expression, with reduced expression of p53, negative for VEGF (not shown in picture), and with high expression of MDM2, $p21^{WAF1/CIP1}$, and TS.

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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, none 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 p21$^{WAF1/CIP1}$, although p21$^{WAF1/CIP1}$ 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 found that among subjects who did not receive 5-FU, those who had a better outcome had MSI cancers (54, 64). Furthermore, considering the MSI group alone (treated and untreated), Ribic et al. found no difference in survival. Thus, the reduced biological aggressiveness of these tumors should not be accounted for the good outcome from 5-FU–based chemotherapy in future studies.

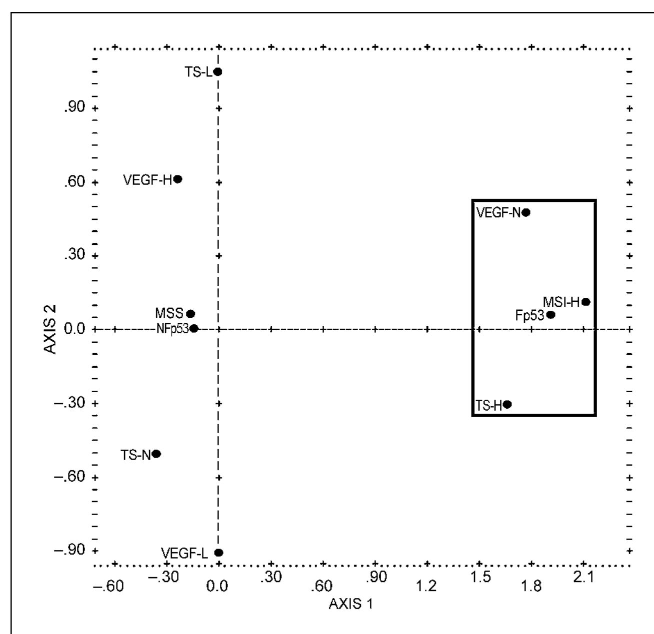

Fig. 3. Plot of the first two axes of multiple correspondence analysis with categories of selected variables: Total inertia was 1.50. The first axis accounted for 34.8% (0.522) and the second axis for 18.7% (0.28) of total inertia. As shown in the box, MSI-H tumors were associated to TS high (*TS-H*), VEGF negative (*VEGF-N*), and functional p53 (*Fp53*), whereas MSS cases were associated to nonfunctional p53 (*NFp53*), high expression of VEGF (*VEGF-H*), and no expression of TS (*TS-N*).

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.

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