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ORIGINAL REPORT

Immunohistochemical Test for MLH1 and MSH2 Expression Predicts Clinical Outcome in Stage II and III Colorectal Cancer Patients

Giovanni Lanza, Roberta Gafà, Alessandra Santini, Iva Maestri, Laura Guerzoni, and Luigi Cavazzini

From the Department of Experimental and Diagnostic Medicine, Section of Anatomic Pathology, University of Ferrara; and the Division of Clinical Oncology, St Anna Hospital, Ferrara, Italy.

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Address reprint requests to Giovanni Lanza, MD, Dipartimento di Medicina Sperimentale e Diagnostica, Sezione di Anatomia Istologia e Citologia Patologica, Università di Ferrara, Via Fossato di Mortara 64/b, 44100 Ferrara, Italy; e-mail: lng@unife.it.

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ABSTRACT

Purpose
To evaluate the prognostic significance of DNA mismatch repair (MMR) status in a large series of stage II and III colorectal cancer patients. The relationship among MMR status, adjuvant chemotherapy, and clinical outcome was also investigated.

Patients and Methods
The study included 718 patients with colorectal adenocarcinoma (393 stage II and 325 stage III) who underwent curative surgical resection. MMR status was determined by immunohistochemical analysis of MLH1 and MSH2 expression. Microsatellite instability (MSI) was assessed in 363 patients using mononucleotide and dinucleotide markers.

Results
One hundred fourteen (15.9%) carcinomas showed abnormal MMR protein (MMRP) expression (96 MLH1 negative and 18 MSH2 negative) and were classified as MMRP negative, whereas 604 tumors demonstrated normal MLH1/MSH2 immunoreactivity (MMRP positive). MLH1/MSH2 expression was closely related to MSI status ($P < .001$) and several clinicopathologic features. Patients with MMRP-negative carcinomas demonstrated a marked reduction in the risk of cancer-related death with respect to patients with MMRP-positive tumors (hazard ratio, 0.2579; 95% CI, 0.1289 to 0.5159). A better clinical outcome for patients with MMRP-negative tumors was observed in both stage II ($P = .0006$) and stage III ($P = .0052$) disease. In stage III disease, the survival advantage conferred by MMRP-negative tumors was more evident among patients treated with surgery alone than among patients who received adjuvant chemotherapy. A nonsignificant trend for survival benefit from adjuvant chemotherapy was observed among patients with MMRP-positive carcinomas but not among those with MMRP-negative carcinomas.

Conclusion
Immunohistochemical testing for MLH1/MSH2 expression provides useful prognostic information for the management of stage II and III colorectal cancer patients.

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INTRODUCTION

Despite great advances in our knowledge of the molecular basis of colorectal cancer, no molecular marker is actually used in the management of the disease. In particular, several genetic changes have been proposed as prognostic indicators, but none of them has been validated for clinical use.[1,2] However, in recent years a growing body of evidence is accumulating that assessment of microsatellite instability (MSI) status provides useful prognostic information in this tumor type.[3]

MSI is characterized by the presence in tumor DNA of widespread alterations in the length of short repeated sequences called microsatellites. According to international convention,[4] colorectal tumors are classified as high-frequency MSI (MSI-H) when instability occurs in at least 30% of the loci examined and as low-frequency MSI (MSI-L) when less than 30% of loci are unstable. Tumors not showing alterations in the length of the DNA sequences studied are classified as microsatellite stable (MSS). MSI-H is the hallmark of hereditary nonpolyposis colorectal cancer and occurs also in 10% to 15% of sporadic large bowel cancers.[5-9] MSI-H colorectal adenocarcinomas develop through a genetic pathway different from that operating in MSS tumors[10-12] and display distinctive pathologic features, such as proximal location, poor differentiation, mucinous and medullary phenotype, and marked peritumoral and intratumoral lymphocytic infiltration.[13-17] In contrast, the clinicopathologic and the molecular

characteristics of MSI-L large bowel tumors do not seem to be different from those of MSS carcinomas, although some specific alterations in MSI-L carcinomas have been described recently.[18,19]

Several investigations demonstrated that MSI-H colorectal carcinomas behave less aggressively than common large bowel tumors. The survival advantage conferred by the MSI-H phenotype was also shown to be independent of tumor stage and other clinical and pathologic variables in studies performed on large series of patients.[16,17,20-27] However, in view of a possible clinical use, the prognostic significance of MSI status in stage II and stage III disease needs to be more precisely defined. In addition, recent evidence suggests that patients with microsatellite unstable colorectal cancers lack a survival benefit from fluorouracil-based adjuvant chemotherapy (FU-AC).[3,28-30] Conflicting results have been reported previously with regard to this topic,[31,32] and the role of MSI status as a predictive factor of benefit from FU-AC requires urgent additional evaluation.

MSI-H is determined by defects in the DNA mismatch repair (MMR) system in the large majority of tumors by inactivation of the *MLH1* and *MSH2* genes.[6,8] In hereditary nonpolyposis colorectal cancer, MSI-H is produced by germline mutations of one of the MMR genes with somatic inactivation of the remaining wild-type allele.[8,9] In sporadic tumors, epigenetic silencing of the *MLH1* gene by promoter methylation is the major mechanism leading to MMR deficiency and MSI-H.[33-35] Genetic or epigenetic inactivation of the *MLH1* and *MSH2* genes is associated frequently with loss of expression of the corresponding protein, and recent investigations demonstrated that immunohistochemical analysis of MLH1 and MSH2 expression specifically identifies MSI-H colorectal carcinomas.[17,36-46] Only a small fraction of MMR-defective tumors, caused by mutations in the *MLH1* gene not associated with the loss of MLH1 immunoreactivity or caused by mutations in the *MSH6* and *PMS2* genes, are not recognized in this way. Therefore, an immunohistochemical test for MLH1 and MSH2 expression represents a rapid, cost-effective, and reliable method for the detection of the large majority of MMR-defective colorectal tumors.

In this study, we evaluated the prognostic significance of MMR status as determined by genetic and immunohistochemical analyses in a large cohort of stage II and stage III colorectal cancer patients. In stage III disease, the relationship among adjuvant chemotherapy, MMR status, and clinical outcome was also investigated.

PATIENTS AND METHODS

The study included 802 consecutive patients with TNM[47] stage II (n = 441) or stage III (n = 361) colorectal adenocarcinoma who underwent curative surgical resection between January 1986 and December 1995 at the St Anna Hospital (Ferrara, Italy). Patients older than 85 years and those with multiple synchronous large bowel carcinomas, idiopathic inflammatory bowel disease, or familial adenomatous polyposis were excluded. Patients who received preoperative radiation therapy and those with a malignant tumor detected within the previous 5 years were also excluded. Of the 802 patients, 41 (20 with stage II and 21 with stage III disease) died postoperatively and 13 (eight stage II and five stage III) were lost to follow-up. An additional 20 patients (14 stage II and six stage III) were excluded because tumor blocks for immunohistochemical analysis were not available. Finally, 10 (1.4%) of the 728 patients examined were excluded from the study because the quality of immunostaining was considered unsatisfactory. The mean age of the remaining 718 patients (393 stage II and 325 stage III; 359 men and 359 women) was 65.0 years (median, 66 years; range, 27 to 85 years). One hundred eighty tumors were located in the right colon (cecum and ascending colon), 102 tumors were located in the transverse colon (including both flexures), 59 tumors were located in the descending colon, 207 tumors were located in the sigmoid colon, and 170 tumors were located in the rectum (comprising the rectosigmoid junction). In most analyses, tumors were subdivided in two anatomic subgroups: carcinomas of the proximal colon (right and transverse colon) and carcinomas of the distal colon (localized distally to the splenic flexure).

The majority of patients were observed at the St Anna Hospital's Division of Clinical Oncology according to a standardized protocol. For the remaining patients, information regarding clinical outcome was obtained from hospital chart review and/or direct telephone interview with the patients' personal physicians. Sixty-five of the 312 patients with stage II colon cancer and 89 of the 236 patients with stage III colon cancer received postoperative FU-AC. Of the 81 patients with stage II rectal cancer, 20 received postoperative radiation therapy and six received postoperative radiation plus chemotherapy. Of the 89 patients with stage III rectal cancer, 17 received postoperative radiation therapy and 33 received postoperative radiation plus chemotherapy. Overall, 71 (18.1%) of the 393 patients with stage II disease and 122 (37.5%) of the 325 patients with stage III disease have been treated with FU-AC. Most patients received a regimen of FU 370 to 400 mg/m^2 plus folinic acid (pure L-form) 100 mg/m^2 daily for 5 days, every 28 days for six cycles.[48] Fifty-eight patients with colon cancer (28 stage II and 30 stage III) were included in a randomized multicenter clinical trial of adjuvant chemotherapy.[49] The mean follow-up in surviving patients was 93.9 months (median, 90.5 months; range, 63 to 144 months).

Histopathologic Evaluation

Tumor type (adenocarcinoma and mucinous adenocarcinoma) and grade of differentiation were determined according to WHO criteria.[50] Carcinomas with a predominant solid growth pattern and mild or moderate nuclear pleomorphism were classified as medullary adenocarcinomas.[51] Lymphocytic infiltration at the advancing tumor margin was evaluated according to Jass et al.[52] Peritumoral Crohn's-like lymphoid reaction was assessed according to Graham and Appelman[53] and classified as present (intense) or absent.[16] Extramural vein invasion was recorded as present only when the finding was considered unequivocal.[16]

Immunohistochemical Analysis

Immunohistochemical analysis of MLH1 and MSH2 expression was performed according to the analytic procedure described previously.[16,41]

Tumors showing complete loss of nuclear MLH1 or MSH2 expression were classified as MLH1 negative or MSH2 negative. Nuclear immunostaining of normal epithelial cells, lymphocytes, and stromal cells served as internal positive controls in each case. Carcinomas with normal expression of MLH1 and MSH2 gene products (ie, presence of nuclear immunostaining in a large proportion of neoplastic cells) were classified as MLH1 positive and MSH2 positive. All tumors were evaluated independently by two pathologists (G.L. and R.G.) without knowledge of clinical data and MSI status.

Immunohistochemical analysis of p53 protein expression was performed as reported in previous studies.[16,51] Tumors showing a proportion of stained nuclei higher than 10% were classified as p53 positive.

Microsatellite Analysis

Microsatellite analysis was performed on samples of tumor and corresponding normal mucosa obtained from fresh surgical specimens, frozen in liquid nitrogen, and stored at −80°C. DNA was extracted by a standard phenol-chloroform procedure. Before DNA extraction, the presence of adequate neoplastic material (at least 60% to 70% of tumor cells) was verified by microscopic examination.

In all the 363 patient samples analyzed, six microsatellite loci (BAT26, BAT40, D18S58, D18S61, D17S855, and D17S786) were examined using a fluorescence-based polymerase chain reaction (PCR) method, as reported previously.[16,41] In addition, in the majority of samples, several of the following microsatellite loci have also been evaluated: BAT25, D2S123, D5S346, D17S250, D18S65, D18S69, D17S796, D17S1176, D8S261, D8S254, and D8S550. PCR products were run in an ABI PRISM 377 DNA sequencer (Perkin-Elmer Applied Biosystems Division, Foster City, CA) and analyzed by the GeneScan 3.1 version software (Perkin-Elmer).

MSI was defined as presence in the tumor DNA of PCR products of abnormal size with respect to the DNA of corresponding normal tissue. According to the guidelines of the International Workshop of Bethesda,[4] tumors showing instability at ≥ 30% of microsatellite loci were classified as MSI-H, tumors demonstrating instability at less than 30% of microsatellite loci were classified as MSI-L, and tumors without detectable MSI were classified as MSS.

DNA Ploidy Analysis

Flow cytometric DNA ploidy analysis was performed in 415 patients using multiple frozen tumor samples, as reported.[54] Carcinomas were classified as DNA diploid or aneuploid according to criteria previously described.[54]

Statistical Analysis

Differences in distributions between the variables examined were assessed with the χ^2 test or the Fisher's exact test, as appropriate. Survival curves were generated according to the method of Kaplan and Meier and compared by the log-rank test. Multivariate analyses were performed with the Cox proportional hazards model. Patients who died as a result of causes unrelated to colorectal cancer were censored at the time of death. The reported *P* values are two-sided and *P* values of less than .05 were considered to indicate statistical significance. All data were analyzed using the SPSS statistical software, Version 8.0 (SPSS Inc, Chicago, IL).

RESULTS

MLH1/MSH2 Expression, MSI Status, and Clinicopathologic Features

Of the 718 colorectal adenocarcinomas examined, 604 (84.1%) showed normal nuclear expression of both MLH1 and MSH2 proteins (MLH1/MSH2 positive), 96 (13.4%) showed complete loss of MLH1 expression with normal MSH2 immunoreactivity (MLH1 negative), and 18 (2.5%) demonstrated complete loss of MSH2 expression with normal MLH1 immunoreactivity (MSH2 negative). In all of the analyses, tumors with abnormal MMR protein expression (MLH1 negative and MSH2 negative, 15.9% of samples) were grouped together

Table 1. Relationship Between Clinicopathologic Features and MLH1/MSH2 Expression in 718 Stage II and Stage III Colorectal Adenocarcinomas

Feature	No. of Patients	MMRP+		MMRP−		*P*
		No. of Patients	%	No. of Patients	%	
Sex						
Male	359	317	52.5	42	36.8	.003
Female	359	287	47.5	72	63.2	
Age, years						
< 50	75	65	10.8	10	8.8	.008
50-70	400	349	57.8	51	44.7	
> 70	243	190	31.4	53	46.5	
Tumor site						
Proximal colon	282	179	29.6	103	90.4	< .001
Distal colon	436	425	70.4	11	9.6	
Tumor stage, TNM						
II	393	320	53.0	73	64.0	.031
III	325	284	47.0	41	36.0	
Tumor type						
Adenocarcinoma	581	528	87.4	53	46.5	< .001
Mucinous adenocarcinoma	107	74	12.3	33	28.9	
Medullary adenocarcinoma	30	2	0.3	28	24.6	
Grade of differentiation						
Well/moderate	551	505	83.6	46	40.4	< .001
Poor	167	99	16.4	68	59.6	
Lymphocytic infiltration at the tumor margin						
Little or none	457	387	64.1	70	61.4	NS
Marked/moderate	261	217	35.9	44	38.6	
Crohn's-like lymphoid reaction*						
Absent	222	174	84.9	48	53.3	< .001
Present	73	31	15.1	42	46.7	
Extramural vein invasion						
Absent	631	526	87.1	105	92.1	NS
Present	87	78	12.9	9	7.9	
DNA ploidy†						
Diploid	119	62	17.9	57	82.6	< .001
Aneuploid	296	284	82.1	12	17.4	
p53 expression‡						
Negative	312	217	40.0	95	88.0	< .001
Positive	339	326	60.0	13	12.0	

Abbreviations: MMR, mismatch repair; MMRP+, MLH1/MSH2 positive; MMRP−, MLH1 or MSH2 mismatch repair protein negative; NS, not significant.
*Evaluated in 295 patients.
†Evaluated in 415 patients.
‡Evaluated in 651 patients.

and indicated as MMR protein (MMRP) negative, whereas MLH1/MSH2-positive carcinomas were indicated as MMRP positive.

Microsatellite analysis was performed in 363 carcinomas (192 stage II and 171 stage III). According to international convention,[4] 271 tumors were classified as MSS (74.7%), 17 were classified as MSI-L (4.7%), and 75 (20.6%) were classified as MSI-H. All of the MSI-L and MSS carcinomas displayed normal MLH1 and MSH2 expression (MMRP positive). Of the 75 carcinomas classified as MSI-H by genetic analysis, 68 (90.7%) were MMRP negative (57 MLH1 negative and 11 MSH2 negative) and seven (9.3%) were MMRP positive ($P < .001$). Therefore, in this series, immunohistochemical analysis recognized more than 90% of MSI-H tumors, with a specificity of 100%.

The relationship between clinicopathologic features and MLH1/MSH2 expression is listed in Table 1. MMRP-negative carcinomas occurred more frequently in women ($P = .003$) and among patients older than 70 years ($P = .008$) with respect to MMRP-positive tumors. In addition, MMRP-negative carcinomas were characterized by proximal location, poor differentiation, mucinous and medullary histology, and marked peritumoral Crohn's-like lymphoid reaction (all $P < .001$). MMRP-negative tumors were also more likely to be stage II than were MMRP-positive cancers (64% *v* 53%; $P = .031$). Finally, the large majority of MMRP-negative carcinomas were p53 negative (88%) and DNA diploid (82.6%), whereas MMRP-positive tumors were prevalently p53 positive (60%; $P < .001$) and DNA aneuploid (82.1%; $P < .001$). Similar results were obtained when the clinicopathologic features of MSI-H and MSI-L/MSS carcinomas were compared (data not shown).

Survival Analyses

Patients with stage III tumors demonstrated reduced disease-specific survival with respect to patients with stage II tumors (Fig 1A; $P < .0001$). One hundred fifty-one (46.5%) of the 325 patients with stage III carcinomas developed distant metastases and/or local recurrence and 142 (43.7%) died as a result of the disease. In contrast, only 72 (18.3%) of the 393 patients with stage II carcinomas developed distant metastases and/or local recurrence and 66 (16.8%) died as a result of the disease (Table 2).

Patients with MMRP-negative tumors showed a better clinical outcome than patients with MMRP-positive carcinomas (Fig 1B; $P < .0001$). Respectively, 10 (8.8%) of the 114 patients with MMRP-negative tumors and 198 (32.8%) of the 604 patients with MMRP-positive cancers died as a result of the disease during the observation period (Table 2). Furthermore, when patients were stratified by TNM stage, the survival advantage for patients with MMRP-negative tumors was clearly evident and statistically significant in both stage II and stage III disease (Figs 1C and 1D; $P = .0006$ and $P = .0052$, respectively). In detail, the 6-year survival rates for patients with stage II MMRP-negative, stage II MMRP-positive, stage III MMRP-negative,

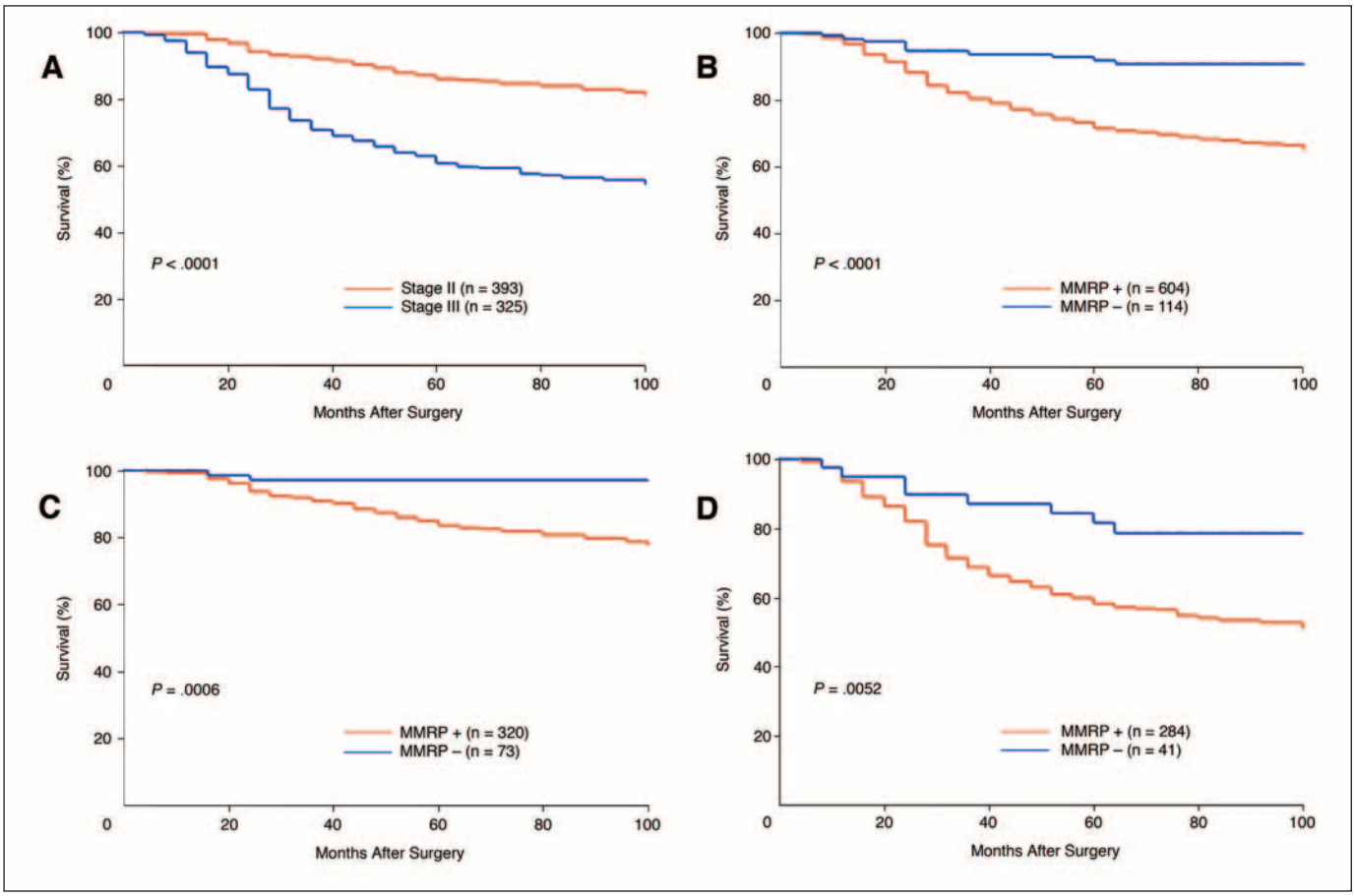

Fig 1. (A) Disease-specific survival of the 718 patients with colorectal cancer included in the study according to TNM stage. Cancer-specific survival of (B) all patients, (C) stage II patients, (D) and stage III patients in relation to MLH1 and MSH2 pattern of immunohistochemical expression (mismatch repair protein [MMRP] +, MLH1/MSH2 positive; MMRP−, MLH1 or MSH2 negative).

Table 2. Cancer-specific Survival in Relation to Clinical, Pathologic, and Molecular Parameters

Feature	No.	Patients Dead as a Result of Recurrent Disease		*P**
		No.	%	
Sex				
Male	359	125	34.8	.0008
Female	359	83	23.1	
Age, years				
≤ 70	475	133	28.0	.1288
> 70	243	75	30.9	
Tumor site				
Proximal colon	282	55	19.5	.0001
Distal colon	436	153	35.1	
Tumor stage, TNM				
II	393	66	16.8	< .0001
III	325	142	43.7	
Tumor type				
Adenocarcinoma	581	174	29.9	.0167†
Mucinous adenocarcinoma	107	33	30.8	
Medullary adenocarcinoma	30	1	3.3	
Grade of differentiation				
Well/moderate	551	146	26.5	.0004
Poor	167	62	37.1	
Lymphocytic infiltration at the tumor margin				
Little or none	457	170	37.2	< .0001
Marked/moderate	261	38	14.6	
Extramural vein invasion				
Absent	631	160	25.4	< .0001
Present	87	48	55.2	
FU-based adjuvant chemotherapy				
Performed	193	51	26.4	.4089
Not performed	525	157	29.9	
p53 expression‡				
Negative	312	77	24.7	.0639
Positive	339	107	31.6	
MLH1/MSH2 expression				
MMRP+	604	198	32.8	< .0001
MMRP−	114	10	8.8	
Microsatellite instability§				
MSI-L/MSS	288	101	35.1	.0002
MSI-H	75	8	10.7	

Abbreviations: FU, fluorouracil; MMRP+, MLH1/MSH2 mismatch repair protein positive; MMRP−, MLH1 or MSH2 negative; MSI-L, low-frequency microsatellite instability; MSS, microsatellite stable; MSI-H, high-frequency microsatellite instability.
*Calculated by log-rank test.
†Medullary adenocarcinoma *v* adenocarcinoma and mucinous adenocarcinoma.
‡Evaluated in 651 patients.
§Evaluated in 363 patients.

and stage III MMRP-positive carcinomas were 97%, 82%, 78%, and 56%, respectively. The survival advantage for patients with MMRP-negative tumors in both stage II and stage III disease was also evident when only tumors of the colon (stage II, $P = .0016$; stage III, $P = .0224$) or only tumors of the proximal colon (stage II, $P = .0043$; stage III, $P = .0121$) were examined.

Among the 363 patients whose tumors have been investigated by genetic analysis, MSI status was significantly related to disease-specific survival. Patients with MSI-H carcinomas showed a better clinical outcome than patients with MSI-L/MSS tumors in all cases ($P = .0002$; Table 2), as well as in stage II and stage III disease analyzed separately ($P = .0059$ and $P = .0375$, respectively; data not shown).

In the group of 203 stage III patients who did not receive FU-AC, patients with MMRP-negative tumors demonstrated a much better clinical outcome than those with MMRP-positive carcinomas (Fig 2A; $P = .0054$). The 6-year survival rates were 79% and 52%, respectively. A nonsignificant trend for better survival of patients with MMRP-negative tumors was observed among the 122 stage III patients treated with FU-AC (Fig 2B; $P = .3177$). However, in this analysis only a small number of patients with MMRP-negative carcinomas was included (n = 9). A trend for a survival benefit from adjuvant chemotherapy was observed among the 284 stage III patients with MMRP-positive tumors, but the difference was not statistically significant ($P = .0776$; Fig 3A). Conversely, among the 41 patients with stage III MMRP-negative carcinomas, no difference in the duration of survival was observed between patients who received adjuvant chemotherapy and those who did not ($P = .9100$; Fig 3B).

In addition to tumor stage and MLH1/MSH2 expression, other clinical and pathologic variables were related significantly to disease-specific survival among the 718 patients included in the study (Table 2). A multivariate analysis according to the Cox proportional hazards model was performed in the whole series of patients including MLH1/MSH2 expression, age at surgery, sex, tumor site, TNM stage, tumor type, grade of differentiation, lymphocytic infiltration at the tumor margin, vein invasion, and FU-AC as covariates (model 1). Patients with MMRP-negative carcinomas demonstrated a marked reduction in the risk of cancer-related death with respect to patients whose tumors showed normal MLH1/MSH2 expression (hazard ratio [HR], 0.2579; 95% CI, 0.1289 to 0.5159; $P = .0001$; Table 3). Similar results were obtained when patients with rectal cancer were excluded from the analysis (HR, 0.2297; 95% CI, 0.1118 to 0.4719; $P = .0001$; data not shown).

A multivariate analysis was also performed in the group of 363 patients whose tumors have been evaluated by microsatellite genotyping. In this analysis (model 2), MSI status was included as covariate (MSI-H *v* MSI-L/MSS), whereas MLH1/MSH2 expression was excluded. In model 2, TNM stage, sex, MSI status, age at surgery, lymphocytic infiltration at the tumor margin, and extramural vein invasion were selected as significant independent predictors of disease-specific survival. Patients with MSI-H tumors exhibited a lower risk of cancer-related death than patients with non–MSI-H carcinomas (HR, 0.3167; 95% CI, 0.1528 to 0.6566; $P = .002$; Table 3).

DISCUSSION

The large majority of previous studies evaluating the prognostic or predictive value of MMR status in colorectal cancer have been performed using microsatellite analysis to assess tumor phenotype. However, genetic analysis of MSI status is time consuming and expensive, and needs specialized equipment. Recently, it has been demonstrated that immunohistochemical analysis of MLH1 and MSH2 expression is a rapid, cost-effective, and accurate method for the assessment of MMR status in colorectal adenocarcinomas.[17,36-46] In this investigation, we used immunohistochemical analysis of MLH1/MSH2 expression to evaluate the prognostic significance of MMR status in a large series of stage II and stage III colorectal cancer patients.

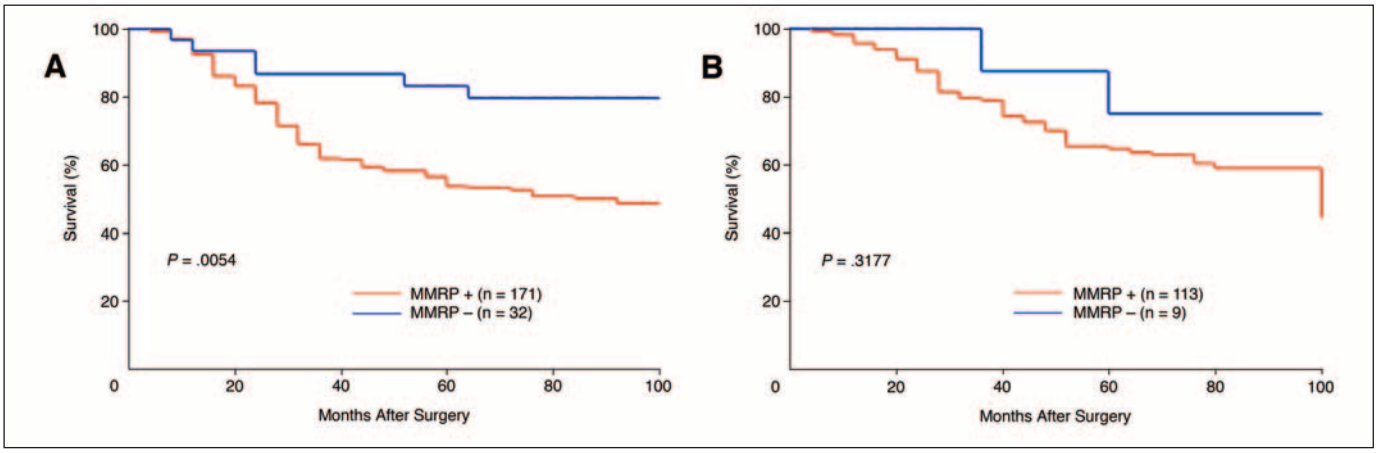

Fig 2. Kaplan-Meier estimates of disease-specific survival among patients with stage III colorectal carcinoma treated with (A) surgery alone or (B) with surgery plus fluorouracil-based adjuvant chemotherapy in relation to MLH1/MSH2 expression (mismatch repair protein [MMRP] +, MLH1/MSH2 positive; MMRP−, MLH1 or MSH2 negative).

In our study, patients whose tumors demonstrated loss of MMR protein expression (MMRP negative) had a better clinical outcome than patients with MMRP-positive tumors in stage II as well as in stage III disease. Moreover, in multivariate analysis, the survival advantage for patients with MMRP-negative carcinomas was independent from several clinical and pathologic parameters. Specifically, patients with stage II MMRP-negative tumors showed an excellent clinical outcome (6-year survival rate, 97%). It is disputed whether patients with stage II colon cancer need to be treated with adjuvant chemotherapy.[55,56] According to our data, stage II patients with MMRP-negative tumors (18.6% of all stage II patients and 43.2% of stage II patients with tumors localized in the proximal colon) should not require any additional treatment after surgical resection.

Patients with MMRP-negative tumors demonstrated a better clinical outcome also in stage III disease. It is important to note that in stage III disease the survival advantage conferred by MMRP-negative carcinomas was clearly evident among patients who did not receive FU-AC (6-year survival rates of 79% and 52% for patients with MMRP-negative and MMRP-positive tumors, respectively). A better survival for patients with MMRP-negative tumors was also observed among stage III patients who received adjuvant chemotherapy, but the difference failed to reach statistical significance. Furthermore, a nonsignificant trend for survival benefit from adjuvant chemotherapy was observed among stage III patients with MMRP-positive tumors, but not among stage III patients with MMRP-negative carcinomas. This last finding should be interpreted with caution, given that only 41 patients with MMRP-negative carcinomas were included in the analysis.

In contrast with early studies,[31,32] recent investigations indicate that colorectal cancer patients with MSI-H tumors do not benefit from FU-AC. Specifically, in a study performed on 570 patients with stage II and III colon cancer who were enrolled onto prospective randomized clinical trials of FU-AC, Ribic et al[29] demonstrated a survival advantage for MSI-H tumors among patients who did not receive adjuvant chemotherapy, but not among patients who received the treatment. Furthermore, a significant beneficial effect of adjuvant chemotherapy on overall survival rate was observed in the group of patients with MSI-L/MSS tumors, whereas among patients with MSI-H carcinomas a trend toward worse clinical outcome for those receiving FU treatment was detected. Likewise, in a series of 204 stage

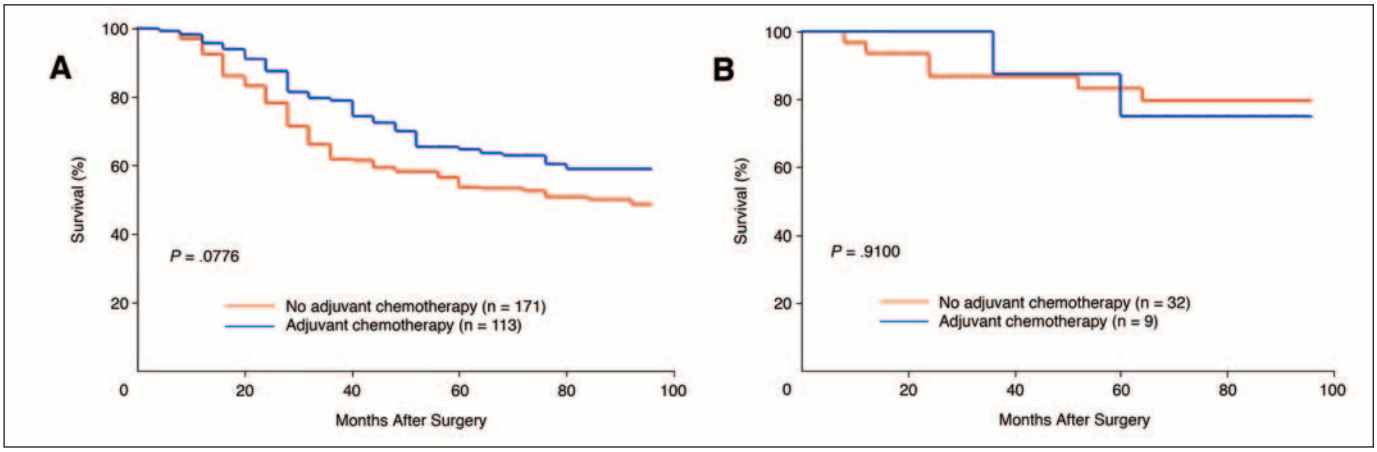

Fig 3. Disease-specific survival of stage III colorectal cancer patients with (A) mismatch repair protein-positive (MMRP+) and (B) MMRP-negative (MMRP−) tumors according to treatment status. Patients with MMRP-positive tumors showed a nonsignificant trend for a survival benefit from fluorouracil-based adjuvant chemotherapy. Among patients with MMRP-negative tumors, no difference in the duration of survival was observed between patients who received adjuvant chemotherapy and those who did not.

Table 3. Multivariate Analysis of Cancer-Specific Survival

Variable	HR	95% CI	*P*
Model 1 (718 patients)*			
Sex, female *v* male	0.7085	0.5352 to 0.9379	.0160
Tumor site, distal colon *v* proximal colon	1.6394	1.1767 to 2.2841	.0035
TNM stage, stage III *v* stage II	2.3978	1.7450 to 3.2949	< .0001
Grade of differentiation, poor *v* well/moderate	1.7755	1.2740 to 2.4742	.0007
MLH1/MSH2 expression, MMRP− *v* MMRP+	0.2579	0.1289 to 0.5159	.0001
Age at surgery, > 70 years *v* ≤ 70 years	1.4643	1.0764 to 1.9918	.0151
FU-based adjuvant chemotherapy, performed *v* not performed	0.6141	0.4331 to 0.8707	.0062
Lymphocytic infiltration at the tumor margin, marked/moderate *v* little/none	0.3993	0.2773 to 0.5750	< .0001
Extramural vein invasion, present *v* absent	1.9050	1.3416 to 2.7050	.0003
Model 2 (363 patients)†			
Sex, female *v* male	0.4890	0.3239 to 0.7382	.0007
TNM stage, stage III *v* stage II	2.2164	1.4399 to 3.4118	.0003
MSI status, MSI-H *v* MSI-L/MSS	0.3167	0.1528 to 0.6566	.0020
Age at surgery, > 70 *v* ≤ 70 years	2.1358	1.4325 to 3.1845	.0002
Lymphocytic infiltration at the tumor margin, marked/moderate *v* little/none	0.4064	0.2488 to 0.6638	.0003
Extramural vein invasion, present *v* absent	1.9330	1.2309 to 3.0355	.0042

Abbreviations: HR, hazard ratio; MMRP+, MLH1/MSH2 positive; MMRP−, MLH1 or MSH2 negative; FU, fluorouracil; MSI, microsatellite instability; MSI-H, high-frequency microsatellite instability; MSI-L, low-frequency microsatellite instability; MSS, microsatellite stable.
*Tumor type was not selected.
†Tumor site, grade of differentiation, FU-based adjuvant chemotherapy, and tumor type were not selected.

II and III colorectal adenocarcinomas, Carethers et al[30] showed a survival advantage for patients receiving FU-AC among those with non–MSI-H tumors but not among those with MSI-H carcinomas. These findings are in accordance with in vitro studies showing that MMR-deficient colon cancer cell lines are less responsive than MMR-proficient cell lines to FU as well as other chemotherapeutic agents.[57-63] Additional investigations are needed to determine the value of MMR status as a predictor of survival benefit from FU-AC, especially in stage III colon cancer.[3] Notwithstanding, our data provide strong evidence that MMR status is a powerful prognostic indicator in stage III patients treated by surgery alone, and that two groups of patients with different clinical outcome are distinguished on the basis of this molecular parameter.

Adjuvant chemotherapy is considered the standard of care for patients with stage III colorectal cancer. However, the clinical course of stage III disease is heterogeneous, and approximately 50% of patients are cured by surgery alone. In our study, stage III patients with MMRP-negative tumors not receiving adjuvant chemotherapy displayed a cancer-specific survival similar to that of patients with stage II disease. If our findings are confirmed in other investigations, the advisability to treat this group of patients with adjuvant chemotherapy should be carefully evaluated, especially if additional markers to select stage III MSI-H tumors with favorable clinical outcome will be available.[64-66]

In agreement with previous studies, we found an excellent correlation between the results obtained by immunohistochemical and genetic analysis in the classification of colorectal tumors according to MMR status. In fact, all of the 288 carcinomas classified as MSS or MSI-L by microsatellite analysis showed normal MLH1/MSH2 expression by immunohistochemistry. Conversely, 68 (90.7%) of the 75 MSI-H carcinomas demonstrated complete loss of MLH1 or MSH2 protein expression. In our study, seven tumors were classified as MSI-H by genetic analysis but showed normal MLH1/MSH2 expression. We performed immunohistochemical analysis of the expression of two other MMR proteins (MSH6 and PMS2) in these seven MSI-H MMRP-positive carcinomas. Four tumors displayed complete loss of MSH6 expression and normal reactivity for PMS2 and one tumor demonstrated complete loss of PMS2 expression (with normal nuclear expression of the MSH6 protein), whereas the remaining two carcinomas were MSH6 and PMS2 positive. Therefore, mutations of the *MSH6* and *PMS2* genes are probably involved in the development of four and one of these cancers, respectively. The two MSI-H adenocarcinomas with normal expression of all of the four MMR proteins tested are most likely generated by mutations in the *MLH1* gene that inactivate the MMR activity, but do not lead to loss of MLH1 immunoreactivity.[46]

In this investigation, MLH1/MSH2 immunoreactivity was demonstrated to be closely related to several pathologic features, such as tumor site, tumor type, grade of differentiation, nodal status, Crohn's-like lymphoid reaction, and also to DNA ploidy pattern and p53 protein expression. These data confirm and extend other investigations in which microsatellite or immunohistochemical analysis was employed to determine the MMR status of the tumors.[13,14,16,25,27,41,67-70] The prognostic significance of MLH1/MSH2 expression in large bowel cancer has been evaluated previously only in a limited number of studies performed on small series of patients.[38,71-74] Here, we demonstrated that immunohistochemical analysis of MLH1/MSH2 expression is suitable for large-scale clinical investigations and provides useful prognostic information for the management of stage II and III colorectal cancer patients.

In conclusion, the results of this study indicate clearly that MMR status is a powerful prognostic indicator in colorectal cancer. In a near future, immunohistochemical analysis of MLH1/MSH2 expression could be introduced as a routine diagnostic test in the pathologic assessment of large bowel tumor specimens.

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Authors' Disclosures of Potential Conflicts of Interest

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Author Contributions

Conception and design: Giovanni Lanza, Roberta Gafà, Luigi Cavazzini
Financial support: Giovanni Lanza
Provision of study materials or patients: Alessandra Santini
Collection and assembly of data: Giovanni Lanza, Roberta Gafà, Alessandra Santini
Data analysis and interpretation: Giovanni Lanza, Roberta Gafà, Iva Maestri, Laura Guerzoni
Manuscript writing: Giovanni Lanza, Roberta Gafà, Luigi Cavazzini
Final approval of manuscript: Giovanni Lanza, Roberta Gafà, Alessandra Santini, Iva Maestri, Laura Guerzoni, Luigi Cavazzini

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Editorial correspondence should be addressed to Daniel G. Haller, MD, *Journal of Clinical Oncology*, 330 John Carlyle St, Suite 300, Alexandria, VA 22314. Telephone: (703) 797-1900; Fax: (703) 684-8720. E-mail: jco@asco.org. Internet: www.jco.org.

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