Reduced Likelihood of Metastases in Patients with Microsatellite-Unstable Colorectal Cancer

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Abstract

Purpose: The outcome of patients with colorectal cancer is more favorable when the tumor exhibits high-frequency microsatellite instability (MSI). Although associated with earlier-stage tumors, MSI has been proposed as an independent predictor of survival. We tested the prognostic value of MSI in a large series of patients diagnosed with colorectal cancer in the last decade.

Experimental Design: The survival of 893 consecutive patients with colorectal cancer characterized by microsatellite status was analyzed. The 89 (10%) patients with MSI cancer were classified according to tumor mismatch repair (MMR) defect, MMR germ-line mutation, *hMLH1* and *p16* promoter methylation, *BRAF* and *K-ras* mutations, and frameshifts of target genes.

Results: The colorectal cancer–specific survival was significantly ($P = 0.02$) better in patients with MSI cancer than in those with stable tumor (MSS). MSI did not predict a significantly lower risk of cancer-related death if tumor stage was included in the multivariate analysis [hazard ratio, 0.72; 95% confidence interval (95% CI), 0.40-1.29; $P = 0.27$]. Instead, MSI was strongly associated with a decreased likelihood of lymph node (odds ratio, 0.31; 95% CI, 0.17-0.56; $P < 0.001$) and distant organ (odds ratio, 0.13; 95% CI, 0.05-0.33; $P < 0.001$) metastases at diagnosis, independently of tumor pathologic features. Molecular predictors of reduced metastatic risk, and then of more favorable prognosis, included *TGFβRII* mutation for all MSI tumors, hMSH2 deficiency for hereditary non-polyposis colorectal cancer, and absence of *p16* methylation for sporadic hMLH1-deficient cancers.

Conclusions: Tumor MSI is a stage-dependent predictor of survival in patients with colorectal cancer. The decreased likelihood of metastases in patients with MSI cancer is associated with specific genetic and epigenetic changes of the primary tumor.

High-frequency microsatellite instability (MSI), reflecting inactivation of the mismatch repair (MMR) genes, is present in nearly all cancers from individuals with hereditary non-polyposis colorectal cancer (HNPCC; refs. 1, 2). Germ-line mutation in a MMR gene, most commonly *hMSH2* and *hMLH1*, is the genetic basis for HNPCC (3, 4). MSI, usually secondary to promoter hypermethylation and silencing of the *hMLH1* gene, is also recognized in 10% to 15 % of sporadic colorectal cancers (5–9).

Besides exhibiting distinctive clinico-pathologic features, such as proximal site, poor differentiation, mucinous cell histotype, and lymphocytic infiltration (10, 11), MSI cancers have a better prognosis than chromosomally unstable but microsatellite stable (MSS) colorectal cancers (12–20). Population-based studies (15, 18, 20), as well as a meta-analysis (21), found that MSI contributed to improved survival by predicting a lower pathologic stage at diagnosis as well as a stage-for-stage predictor of more favorable outcome. Despite this evidence, several issues need to be settled before adopting MSI as a prognostic variable in the clinical management of colorectal cancer.

Data supporting the prognostic benefit of tumor instability are almost exclusively derived from series of patients diagnosed with colorectal cancer in the 1980s or in the early 1990s, when modalities of cancer staging were far less accurate. Therefore, inadequate staging might have affected the interpretation of the prognostic advantage of MSI cancers. Consistent with this hypothesis, the only large prospective and population-based study conducted on patients diagnosed in the last decade failed to observe any significant stage-independent survival advantage for carriers of mutations in MMR genes (22).

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5-Fluorouracil (5-FU) adjuvant therapy may also act as a confounding factor when evaluating the prognostic role of MSI in patients with colorectal cancer diagnosed and treated in different eras. Some authors reported a survival advantage or at least a very good survival in 5-FU–treated patients with MSI cancer (16, 17), whereas others claimed that only patients with MSS cancer benefit from 5-FU adjuvant therapy (19, 20, 23, 24). If the latter were true, the widespread chemotherapeutic approach of the last decade might have attenuated the overall prognostic difference between MSI and MSS tumors.

Finally, the wide spectrum of molecular changes determining or reflecting the genetic instability of MSI tumors may also have prognostic implications. The recognition of a more favorable outcome for HNPCC patients (13, 20) has recently challenged the concept that the clinical outcome of carriers of MMR gene germ-line mutations and that of patients with sporadic MSI cancer are equivalent (25). In addition, a better survival of patients with *TGFβRII*-mutated cancer (17, 26) and the absence of deaths in a small series of patients with MSI cancer negative for markers of widespread CpG island methylation (27) have been reported.

The aims of the study were to assess tumor microsatellite status and patient survival in a large, mono-institutional series of consecutive and unselected patients having their colorectal cancer diagnosed in the last decade and to search for prognostic molecular markers of MSI cancer.

Patients and Methods

Study population and retrieval of clinico-pathologic data. The study included consecutive and unselected Caucasian patients undergoing resective surgery for colorectal cancer at our Institution between January 1, 1997 and February 28, 2005. Patients were excluded from the study if (*a*) pathologic examination did not confirm a tumor invading at least the submucosa (pT_1 or higher); (*b*) the tumor was a local recurrence of previously resected colorectal cancer; and (*c*) the diagnosis had been the result of screening or surveillance in individuals from families with proven or suspected hereditary colon cancer syndromes. The study was approved by the Ethical Committee of the Istituto Clinico Humanitas, and the informed consent of the patients to the treatment of their personal data was obtained by the referring physician or by other clinicians involved in the study.

A clinical database was prepared by investigators (D.G. and C.C.) blind to the results of cancer genetic testing. Pathologic tissue specimens were reviewed by a single pathologist (R.M.) who was also unaware of molecular data. Tumor pathologic staging, histopathologic typing, tumor grade, and presence or absence of extramural vein invasion were assessed (28, 29). Tumor clinicopathologic staging was finally assessed by combining histopathologic findings, surgical records (including intraoperative liver ultrasonography), and perioperative imaging (abdominal computerized tomography and chest X-rays in all patients). Demographics and complete clinical data at diagnosis were made available at hospital Intranet resources. An accurate family history, aimed at recognizing the Amsterdam clinical criteria for HNPCC (AC-II; ref. 30), was obtained from all the patients. Information on postoperative therapy of patients followed elsewhere was available in all cases. Chemotherapy was always administered on clinical grounds and not in the context of prospective trials.

The overall survival was calculated from diagnosis until death, or until data were censored, as of September 30, 2006. At this date, each patient was confirmed to be alive by direct phone call or by formal inquiry at the local registry of vital statistics.

To maximize any possible association between biological variables and colorectal cancer prognosis, the disease-specific survival was also assessed. For this analysis, we considered as disease-related events only deaths of patients who had imaging-documented colorectal cancer progression and no other obvious cause of death. Cardiovascular accidents, trauma, infectious disease, or progression of cancer other than colorectal cancer were always excluded by reviewing the hospital records or by interviewing the reference physician or a family member. Data from patients deemed to have died from causes other than colorectal cancer as well as data from 10 patients (1.1%) whose in-hospital death was due to post-surgical complications were censored at the time of death.

Assessment of MSI and molecular subtyping of MSI cancers. Coded sections of paraffin-embedded colorectal cancer tissue were sent from the pathologist to the Research Laboratory. If tumor cells did not account for at least 50% of the cells present in the section, tumor microdissection was done. MSI assignment was based on the analysis of repeats in mononucleotides. Differently from protocols including dinucleotide markers (11, 31), this method has been specifically validated for identification of tumors with high levels of MSI (32–34). After DNA extraction by proteinase-K digestion and phenol-chloroform purification, amplification of the *BAT26* locus with fluoresceinated primers (2, 35) was followed by capillary-gel electrophoresis (ABI PRISM 310 DNA Sequencer, Perkin-Elmer). To check for MSI possibly due to homozygous deletions in *hMSH2*, tumors from patients fulfilling the AC-II criteria ($n = 29$) and those from patients ages ≤50 years ($n = 90$) were also tested for *BAT25* instability (36). However, no *BAT26*-stable/*BAT25*-unstable tumor was found.

MSI tumors were investigated for MMR protein defects by immunohistochemistry. Nuclear expression of hMLH1 (G-168 monoclonal antibody, PharMingen) and of hMSH2 (clone FE 11, Oncogene Sciences) was initially tested. In tumors expressing both hMLH1 and hMSH2, the expression of hMSH6 (clone 44, Transduction Laboratories) was also done according to previously described methods (37). Immunohistochemical staining was visualized by the avidin-biotin method (Vectastain, Vector Laboratories). Eight MSS tumors from AC-II–positive patients were also tested for protein expression but showed no MMR defect.

In MSI tumors, the absence or the presence of frameshift mutations at coding mononucleotide repeats of *TGFβRII*, *BAX*, *hMSH3*, *hMSH6*, *TCF4*, *MBD4*, and *CASP-5* was assessed by PCR and capillary electrophoresis (35). Mutations of *K-RAS* codon 12 and 13 and of *BRAF* V600E were investigated by PCR-RFLP (38). The DNA methylation status of *hMLH1* and *p16* promoters was determined by methylation-sensitive PCR based upon DNA treatment with sodium bisulfite and amplification with primers specific for methylated and unmethylated DNA (39).

Sequencing of *hMSH2* or of *hMLH1*, according to the tumor MMR protein defect, was done in all patients with MSI cancer. Exons and intron/exon boundaries of *hMSH2* and *hMLH1* were amplified according to previously described techniques (40). Mutation-negative patients were further tested by multiplex ligation-dependent probe amplification (MLPA kit: Medical Research Council-Holland, Amsterdam, the Netherlands), but no MMR germ-line large deletion was found.

Statistical analysis. The association between clinico-pathologic features and MSI cancers, hereditary or sporadic, was analyzed with a Fisher's exact test for categorical variables and with an unpaired Student's test for age. The association of microsatellite status with metastases to regional lymph nodes or distant organs was evaluated with a multivariate logistic regression including all pathologic variables. The univariate association of metastases with subgroups of MSI cancers with distinct molecular features was assessed by the Fisher's test. Survival curves were drawn according to the Kaplan-Meyer method, and univariate survival distributions were compared using the log-rank test. To test the microsatellite status as a predictor of

cancer-specific risk of death, we did a multivariate analysis according to the Cox proportional-hazards model. All candidate prognostic factors were initially entered into the model, but nonsignificant ($P > 0.1$) variables other than microsatellite status were subsequently rejected (step-down variable selection). In addition, a model in which tumor stage had been arbitrarily excluded from analysis was created. Two-sided $P < 0.05$ was considered statistically significant.

Results

Clinico-pathologic features according to microsatellite status. Of 893 consecutive primary colorectal cancer, 89 (10%) tumors exhibited MSI. Twenty-nine (3.2%) MSI tumors were classified as HNPCC, being from carriers of MMR gene

Table 1. Demographics and clinico-pathological features of 893 consecutive patients with colorectal cancer characterized by microsatellite status

	MSS, *n* (%)	MSI, *n* (%)	*P*	MSI HNPCC, *n* (%)	MSI sporadic, *n* (%)	*P*
All patients	804 (90.0)	89 (10.0)		29 (32.6)	60 (67.4)	
Age at diagnosis (y), mean ± SD	65.1 ± 11.1	65.9 ± 14.7	0.61	53.3 ± 15.5*	72.0 ± 9.7*	<0.001
Gender						
Male	465 (57.8)	54 (60.7)	0.65	19 (65.5)	35 (58.3)	0.64
Female	339 (42.2)	35 (39.3)		10 (34.5)	25 (41.7)	
Tumor site						
Distal colon †	338 (42.0)	12 (13.5)	<0.001	8 (27.6)	4 (6.7)	0.02
Proximal colon †	226 (28.1)	72 (80.9)		19 (65.5)	53 (88.3)	
Rectum	240 (29.9)	5 (5.6)		2 (6.9)	3 (5.0)	
Tumor stage						
I	146 (18.2)	13 (14.6)	<0.001	8 (27.6)	5 (8.3)	0.12
II	204 (25.4)	42 (47.2)		11 (37.9)	31 (51.7)	
III	237 (29.4)	27 (30.3)		8 (27.6)	19 (31.7)	
IV	217 (27.0)	7 (7.9)		2 (6.9)	5 (8.3)	
Tumor invasion ‡						
T_1	50 (6.2)	2 (2.2)	0.08	1 (3.4)	1 (1.6)	0.08
T_2	131 (16.3)	11 (12.4)		7 (24.1)	4 (6.7)	
T_3	537 (66.8)	60 (67.4)		15 (51.8)	45 (75.0)	
T_4	86 (10.7)	16 (18.0)		6 (20.7)	10 (16.7)	
Nodal status§						
N_0	381 (47.4)	56 (62.9)	0.01	19 (65.5)	37 (61.7)	0.24
N_1	233 (28.0)	23 (25.9)		9 (31.0)	14 (23.3)	
N_2	190 (23.6)	10 (11.2)		1 (3.5)	9 (15.0)	
Tumor grade‖						
G_1	63 (7.8)	10 (11.3)	<0.001	6 (20.7)	4 (6.7)	0.08
G_2	568 (70.7)	44 (49.4)		15 (51.7)	29 (48.3)	
G_3	127 (15.8)	35 (39.3)		8 (27.6)	27 (45.0)	
NA	46 (5.7)	0		0	0	
Tumor cell type						
Adenocarcinoma¶	757 (94.2)	67 (75.3)	<0.001	24 (82.8)	43 (71.7)	0.54
Mucinous	40 (5.0)	12 (13.5)		3 (10.3)	9 (15.0)	
Signet ring	5 (0.6)	7 (7.9)		2 (6.9)	5 (8.3)	
Medullary	2 (0.2)	3 (3.3)		0	3 (5.0)	
Tumor invading extramural vein						
No	606 (75.4)	67 (75.3)	0.98	22 (45.9)	45 (74.0)	0.93
Yes	198 (24.6)	22 (24.7)		7 (24.1)	15 (25.0)	
5-FU therapy						
Stage II						
Yes	98 (48.0)	14 (33.3)	0.09	6 (54.5)	8 (25.8)	0.14
No	106 (52.0)	28 (66.7)		5 (45.5)	23 (74.2)	
Stage III						
Yes	180 (75.9)	17 (63.0)	0.16	6 (75.0)	11 (57.9)	0.66
No	57 (24.1)	10 (37.0)		2 (25.0)	8 (42.1)	
Stage IV						
Yes	134 (61.8)	4 (57.1)	0.81	2 (100)	2 (40.0)	0.42
No	83 (38.2)	3 (42.9)		0	3 (60.0)	

NOTE: Sporadic, Amsterdam criteria II not fulfilled and no germ-line mutation.
t test (for age) or Fisher's exact test for comparative analysis of variables.
*$P < 0.001$ vs. MSS.
†To splenic flexure.
‡T_1, tumor invading submucosa; T_2, invading muscolaris propria; T_3, invading through the muscularis propria; T_4, invading adjacent organs or perforating visceral peritoneum.
§N_0, no lymph node involved [number of analyzed nodes (mean ± SE): MSS, 20.5 ± 0.62; MSI, 22.6 ± 1.51; $P = 0.21$, *t* test]; N_1, 1-3 positive nodes; N_2, ≥4 positive nodes.
‖G_1, well; G_2, moderately; G_3, poorly differentiated; NA, not assessable in rectal cancers pre-treated with radiotherapy.
¶Not otherwise specified.

germ-line pathogenic mutations (n = 23: 11 for *hMSH2* and 12 for *hMLH1*) or from patients fulfilling the Amsterdam criteria in the absence of a detectable mutation (n = 6: 4 for *hMSH2*, 1 for *hMLH1*, and 1 for *hMSH6*; Supplemental Data 1). The remaining 60 (6.7%) MSI cancers were categorized as sporadic. Table 1 shows the main clinico-pathologic features of patients with MSS cancer compared with those of patients with MSI cancer, either hereditary or sporadic. MSI cancers had a significantly lower stage at the time of diagnosis ($P < 0.001$). Fifty-five of 89 (61.8%) MSI cancers and 350 of 804 (43.5%) MSS cancers had no lymph node or distant metastases ($P = 0.001$). Among metastatic tumors (stage III and IV), stage IV was more frequent ($P = 0.002$) in MSS (217 of 454, 47.8%) than in MSI (7 of 34, 20.6%) cancers. The overall stage distribution was not significantly different between HNPCC and MSI sporadic cancers. MSI cancers tended to be more locally invasive than MSS cancers, had a more proximal site, were less differentiated, and more frequently had a mucinous, signet ring or medullary histotype. No significant difference in rates of chemotherapy administration was observed between patients with MSI cancer and those with MSS tumor.

Molecular changes in hereditary (HNPCC) and sporadic MSI cancers. Fifteen of 18 (83.3%) hMSH2-deficient cancers were HNPCC. Of 68 hMLH1-deficient cancers, 13 (19.1%) were HNPCC, and 55 (80.9%) were sporadic cancers. *K-ras* was more frequently ($P < 0.001$) mutated in HNPCC (14 of 29,48.3%) than in MSI sporadic cancers (5 of 60, 8.3%). *BRAF* mutation was detected in 34 of 60 (56.7%) sporadic cancers but in no HNPCC ($P < 0.001$). *hMLH1* promoter methylation was more frequent ($P < 0.001$) in MSI sporadic cancers (47 of 60,78.3%) than in HNPCC (12 of 29, 41.4%). HNPCC exhibited mutations of *TCF4* (17 of 29, 58.6%) more often than did MSI sporadic cancers (15 of 60, 25%; $P = 0.004$; Supplemental Data 2).

Likelihood of metastases at diagnosis. Multivariate logistic regression analysis showed a decreased likelihood of lymph node [odds ratio (OR), 0.31; 95% confidence interval (95% CI), 0.17-0.56; $P < 0.001$] and distant organ (OR, 0.13; 95% CI, 0.05-0.33, $P < 0.001$) metastases in patients with MSI cancer versus those with MSS tumor, which was independent of any pathologic feature (Table 2).

The likelihood of metastases at diagnosis was variably reduced in subsets of primary unstable cancers with distinctive molecular changes (Table 3). MSI cancers with no *TGFβRII* mutation or lacking the hMSH6 protein had a likelihood of lymph node or distant organ metastases not significantly different from that of MSS tumors. The rate of nodal metastases in the 23 patients with ascertained MMR germ-line mutation was not significantly different from that of patients with MSS cancer. The likelihood of lymph node metastases was more markedly reduced in patients with hMSH2-deficient tumor (OR, 0.26; 95% CI 0.08-0.79, $P = 0.02$) than in those with hMLH1-deficient cancer (OR, 0.59; 95% CI, 0.36-0.98, $P = 0.04$). Finally, among patients with sporadic MSI and hMLH1-defective cancer, only patients whose cancer was not methylated for *p16* had a significantly decreased likelihood of lymph node metastases (OR, 0.18; 95% CI, 0.05-0.63, $P = 0.007$).

Survival analysis. A total of 319 (35.7%) deaths were registered among the 893 patients over a mean follow-up of 4.35 ± 0.09 years (MSS: 294 of 804, 36.6%; MSI: 25 of 89,

Table 2. Microsatellite status and pathological features of the primary tumor as predictive factors for metastases at diagnosis in 893 patients with colorectal cancer (multivariate logistic regression analysis)

	Lymph node metastases				Distant organ metastases			
	No	**Yes**	**OR (95% CI)**	***P***	**No**	**Yes**	**OR (95% CI)**	***P***
Microsatellite status								
MSS	381	423	1.00 (reference)	<0.001	587	217	1.00 (reference)	<0.001
MSI	56	33	0.31 (0.17-0.56)		82	7	0.13 (0.05-0.33)	
Site								
Distal	163	187	1.00 (reference)		248	102	1.00 (reference)	
Proximal	146	152	0.80 (0.55-1.16)	0.24	224	74	0.74 (0.50-1.11)	0.15
Rectum	128	117	0.99 (0.67-1.48)	0.99	197	48	0.58 (0.37-0.92)	0.02
Tumor invasion								
T_1	44	8	1.00 (reference)		51	1	1.00 (reference)	
T_2	117	25	1.18 (0.48-2.94)	0.72	136	6	2.02 (0.23-17.6)	0.52
T_3	252	345	6.14 (2.71-13.9)	<0.001	432	165	14.1 (1.89-105)	0.01
T_4	24	78	11.2 (4.33-28.8)	<0.001	50	52	37.5 (4.84-291)	<0.001
Tumor grade								
G_1	55	18	1.00 (reference)		70	3	1.00 (reference)	
G_2	312	300	1.76 (0.94-3.29)	0.08	459	153	4.97 (1.48-16.7)	0.009
G_3	41	121	4.03 (1.87-8.69)	<0.001	102	60	5.81 (1.63-20.7)	0.007
NA	29	17	1.16 (0.46-2.92)	0.76	38	8	4.97 (1.15-21.6)	0.03
Tumor cell type								
Adenocarcinoma	415	409	1.00 (reference)		623	201	1.00 (reference)	
Mucinous	18	34	0.96 (0.47-1.96)	0.90	35	17	1.25 (0.61-2.55)	0.54
Signet ring	2	10	3.97 (0.57-27.6)	0.16	9	3	0.83 (0.17-4.13)	0.82
Medullary	2	3	0.66 (0.08-5.19)	0.70	2	3	7.87 (0.89-69.9)	0.06
Extramural vein invasion								
No	390	283	1.00 (reference)	<0.001	546	127	1.00 (reference)	<0.001
Yes	47	173	3.49 (2.37-5.15)		123	97	2.44 (1.69-3.52)	

NOTE: OR < 1.00 represents a decreased likelihood of metastases, whereas OR > 1.00 represents an increased likelihood of metastases.

Table 3. Likelihood of metastases at diagnosis in 89 patients with MSI colorectal cancer according to distinctive molecular changes in the primary tumor (univariate analysis, Fisher's exact test)

	Lymph node metastases				Distant organ metastases			
	No	Yes	OR (95% CI)	*P*	No	Yes	OR (95% CI)	*P*
MSS, reference	381	423	1.00		587	217	1.00	
MSI by								
TGFβRII mutation								
Yes	49	25	0.46 (0.28-0.76)	0.002	70	4	0.15 (0.05-0.43)	<0.001
No*	7	8	1.03 (0.37-2.86)	0.95	12	3	0.68 (0.19-2.42)	0.55
MMR germ-line mutation								
Yes	14	9	0.58 (0.25-1.35)	0.21	22	1	0.12 (0.02-0.92)	0.04
No	42	24	0.51 (0.31-0.87)	0.01	60	6	0.27 (0.11-0.63)	0.003
MMR protein defect								
HMSH2	14	4	0.26 (0.08-0.79)	0.02	18	0	NA	0.01
hMLH1	41	27	0.59 (0.36-0.98)	0.04	63	5	0.21 (0.08-0.54)	0.001
HMSH6	1	2	1.80 (0.16-19.9)	0.63	1	2	5.41 (0.49-60.0)	0.17
p16 methylation †								
No	15	3	0.18 (0.05-0.63)	0.007	18	0	NA	0.01
Yes	18	19	0.85 (0.44-1.65)	0.64	33	4	0.33 (0.11-0.94)	0.04

NOTE: OR < 1.00 represents a likelihood of metastases lower than in the reference group (patients with MSS cancer).
Abbreviation: NA, not applicable.
*The 15 cancers exhibiting wild-type *TGFBRII* were distributed as follows: 3/3 hMSH6 deficient, 2/15 hMSH2-deficient HNPCC, 3/13 hMLH1-deficient HNPCC, 4/37 sporadic hMLH1 deficient/*p16* methylated, and 3/18 sporadic hMLH1 deficient/*p16* unmethylated.
† Data refer to the 55 sporadic hMLH1-deficient MSI cancers.

28.1%; $P = 0.13$ at Fisher's test). If patients older than 75 years at diagnosis were excluded from analysis, the overall number of deaths was significantly different in the two subgroups (MSS: 224 of 647, 34.6%; MSI: 12 of 62, 19.4%; $P = 0.01$). A colorectal cancer–related death was assessed in 252 (28.2%) of all patients (MSS: 239 of 804, 29.7%; MSI: 13 of 89, 14.6%; $P = 0.001$).

Also at Kaplan-Meier analysis, the overall survival of patients with MSS was not significantly different from that of patients with MSI cancer ($P = 0.35$), unless older (>75 years) patients were excluded ($P = 0.05$). Differently, the disease-specific survival of all patients with MSI colorectal cancer was significantly ($P = 0.02$) better than that of all patients with MSS cancer (Fig. 1).

The comparative analysis of cancer-related survival of patients with molecular subsets of MSI cancer (Fig. 2) reflected the differential rate of metastatic disease. First, only patients with *TGFβRII*-mutated MSI cancer had a better survival than patients with stable tumor. As to HNPCC/sporadic classification, neither the survival of all patients with molecular diagnosis of HNPCC ($P = 0.09$) nor that of all patients with hMLH1-deficient sporadic cancer ($P = 0.08$) was significantly better than that of patients with MSS tumor (curves not shown). However, the subset of HNPCC patients with hMSH2 mutation and the subgroup of patients with *p16*-unmethylated hMLH1-deficient sporadic cancer had a statistically significant survival advantage over patients with MSS tumor.

After stratification by tumor stage (Fig. 3), no advantage in disease-specific survival for patients with MSI cancer was maintained. In particular, no effect of MSI on the estimated 5-year survival (percent ± SE) of patients with stage II (MSI, 90.7 ± 5.1 versus MSS, 89.8 ± 2.6) and stage III cancer (70.9 ± 11.4 versus 73.5 ± 3.5) was observed. Conversely, as patients were subgrouped according to the depth of tumor invasion (pT), a better survival of patients with MSI cancer was seen among patients with deeply invading cancer (pT_3: 81.5 ± 6.0 versus 63.4 ± 2.3, $P = 0.02$; pT_4: 53.3 ± 18 versus 34.3 ± 6.0, $P = 0.07$; Supplemental Data 3).

At Cox univariate analysis (Supplemental Data 4), MSI was associated with a significantly reduced risk of cancer-related death [hazard ratio (HR), 0.53; 95% CI, 0.30-0.92; $P = 0.03$]. Tumor stage, invasion, grading, and extramural vein invasion were also highly significant ($P < 0.001$) predictors of outcome. Postoperative 5-FU therapy was associated with a better survival in patients with stage III (HR, 0.55, 95% CI, 0.32-0.97, $P = 0.04$) and stage IV (HR, 0.48, 95% CI, 0.35-0.65, $P < 0.001$) cancer. Upon multivariate analysis (Table 4), MSI was found to predict a better disease-specific survival (HR, 0.30; 95% CI, 0.16-0.54; $P < 0.001$) only if the tumor stage variable was excluded from the analysis. In such a model, female gender, tumor invasion, tumor grade, extramural vein invasion, and 5-FU therapy were other independent predictors of better outcome.

Discussion

This study was aimed to investigate the survival of patients with MSI cancer, compared with that of patients with MSS tumor, in a large mono-institutional series of consecutive and unselected patients with colorectal cancer. The 10% prevalence of MSI observed in our study is lower than that of previous series enriched with clinically suspected HNPCC (20) or preselected by age (15), whereas it is almost identical to the prevalence of MSI tumors detected in large and unselected cohorts of colorectal cancer patients (8, 9, 41). In addition, differences in the MSI assignment protocol (i.e., the number of mononucleotides used) might theoretically account for a somewhat lower prevalence of MSI. However, the use of *BAT26* alone has been recently shown to identify nearly all MSI colorectal cancer (32, 34, 41).

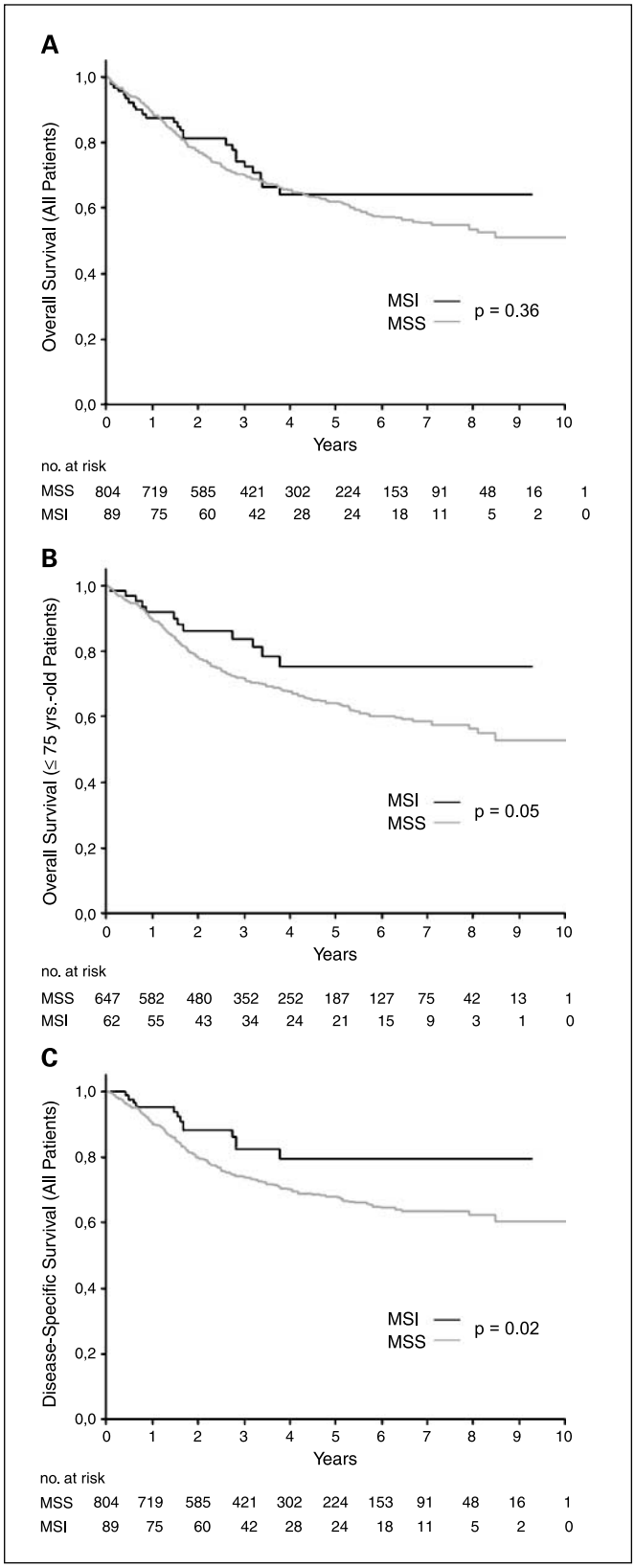

Fig. 1. Survival of patients with colorectal cancer according to tumor microsatellite status (MSS, stable; MSI, unstable). Kaplan-Meier curves including tumor stages I to IV. *A,* overall survival of all patients. *B,* overall survival of patients younger than 76 y at time of diagnosis. *C,* disease-specific survival of all the investigated patients.

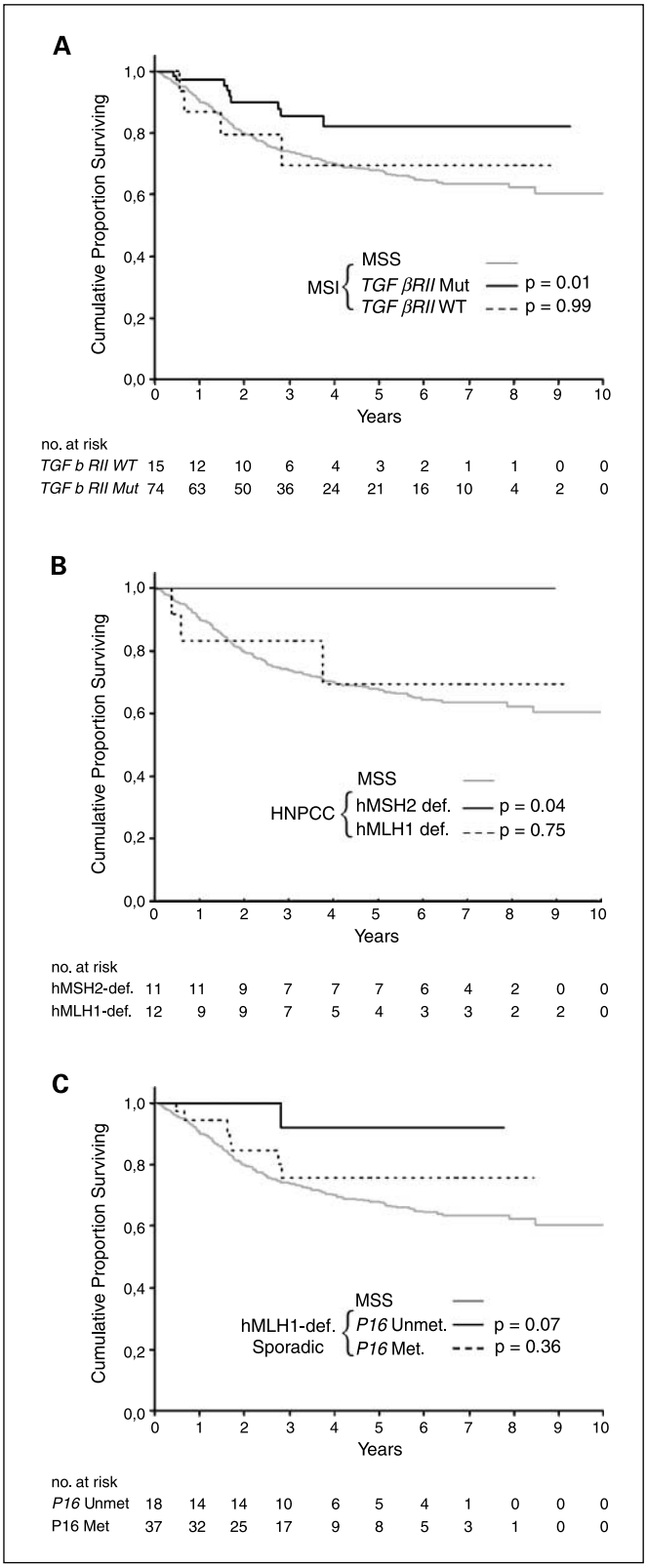

Fig. 2. Disease-specific survival of patients with molecular subsets of MSI colorectal cancer. Kaplan-Meier curves including tumor stages I to IV. Patients (n = 804) with MSS cancer were used as a reference. *A,* patients with MSI cancer, with (n = 74) or without (n = 15) *TGFβRII* mutation. *B,* patients with hMSH2-deficient (n = 11) or hMLH1-deficient (n = 12) HNPCC with an ascertained MMR germ-line mutation. *C,* patients with hMLH1-deficient sporadic cancer (n = 55), *p16* methylated (n = 37) or *p16* unmethylated (n = 18).

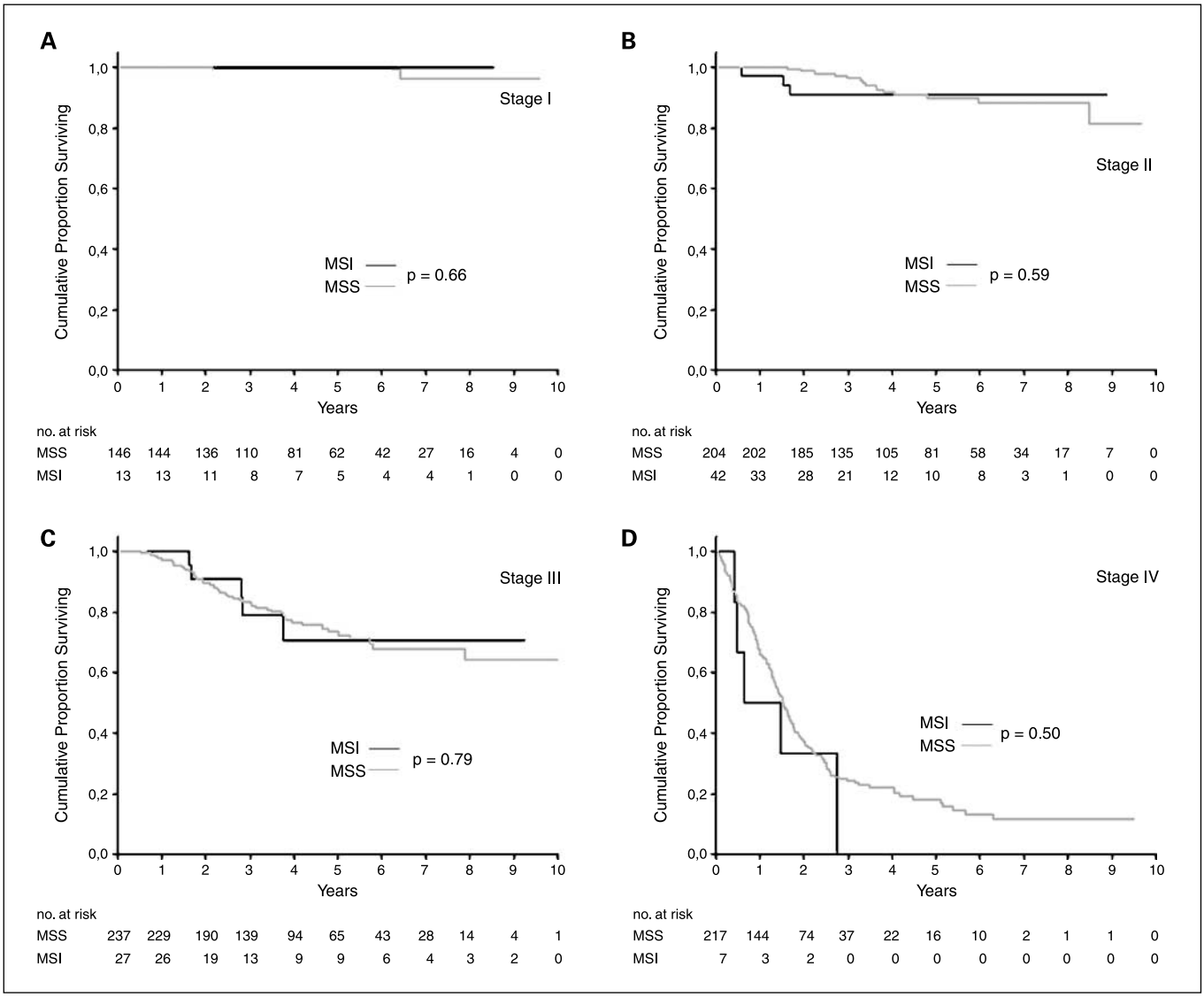

Fig. 3. Disease-specific survival of 893 patients with colorectal cancer stratified by American Joint Committee on Cancer stage (I-IV), according to tumor microsatellite status (MSS, stable; MSI, unstable). No significant difference in survival was observed between patients with MSI cancer and those with MSS tumor at the same stage. *A,* stage I. *B,* stage II. *C,* stage III. *D,* stage IV.

The analysis of survival conducted in our colorectal cancer patients according to the presence or the absence of tumor MSI has provided important findings. First, the overall survival advantage of patients with MSI tumor can be obscured by the fact that many patients develop a sporadic MSI colorectal cancer at a very old age when several competing risks of death are present. Second, the clear advantage in disease-specific survival of patients with MSI cancer is dependent on the earlier stage at which these cancers are diagnosed. Finally, subsets of MSI cancers with different molecular changes may have different biological behavior and prognosis.

Previous population-based studies, although recognizing the lower staging of MSI cancers, have proposed MSI as a stage-independent predictor of better survival (15, 18, 20). In contrast, we found that tumor MSI can predict a lower risk of cancer-related death regardless of standard prognostic factors including tumor local invasion (pT) but not independently of the whole tumor-node-metastasis classification, which takes into account the nodal status and the absence/presence of distant organ metastases. Given that patients with MSI tumor have a decreased likelihood of metastases at diagnosis, these findings clearly indicate that the reduced metastatic potential of the primary tumor is the key mechanism for the survival advantage of patients with unstable colorectal cancer.

The stage-adjusted survival of our patients with MSI colorectal cancer was in the range of those reported by single discordant studies, whereas the cancer-specific 5-year survival rate of patients with stage II (89.8 $\pm$ 2.6%) and stage III (73.5 $\pm$ 3.5%) MSS colorectal cancer was considerably higher than that reported in older series (stage II, ~80%; stage III, ~60%, refs. 18, 20). The somewhat shorter follow-up period of our study, due to the recruitment of patients diagnosed in the last decade, is not likely to account for such a difference. If one accepts that MSS tumors respond better than MSI cancers to 5-FU therapy (19, 24), the extensive use of adjuvant treatment might have selectively improved the survival of patients with

Table 4. Multivariate analysis of predictive factors for disease-specific risk of death in 893 patients with colorectal cancer characterized by microsatellite status

	Cox proportional-hazards models*			
	Model A		Model B	
	HR (95 % CI)	*P*	HR (95 % CI)	*P*
Microsatellite status				
MSS	1.00 (reference)	0.27	1.00 (reference)	<0.001
MSI	0.72 (0.40-1.29)		0.31 (0.17-0.54)	
Gender				
Male	1.00 (reference)	0.08	1.00 (reference)	0.01
Female	0.79 (0.61-1.03)		0.72 (0.55-0.93)	
AJCC stage				
I	1.00 (reference)			
II	2.11 (0.36-12.4)	0.41	Variable excluded from analysis	
III	7.99 (1.43-44.6)	0.02		
IV	52.2 (9.59-295)	<0.001		
Tumor invasion				
T_1-T_2	1.00 (reference)		1.00 (reference)	
T_3	3.71 (1.35-10.2)	0.01	12.2 (5.35-27.7)	<0.001
T_4	4.75 (1.66-13.6)	0.004	23.6 (9.91-56.2)	<0.001
Tumor grade				
G_1	1.00 (reference)		1.00 (reference)	
G_2	1.46 (0.63-3.35)	0.38	2.48 (1.09-5.62)	0.03
G_3	2.70 (1.14-6.39)	0.02	4.27 (1.83-9.93)	<0.001
NA	2.58 (0.93-7.08)	0.07	2.60 (0.96-7.08)	0.06
Tumor invading extramural vein				
No	1.00 (reference)	<0.001	1.00 (reference)	<0.001
Yes	1.92 (1.48-2.48)		2.57 (1.97-3.34)	
5-FU therapy				
No	1.00 (reference)	<0.001	1.00 (reference)	<0.001
Yes	0.39 (0.30-0.50)		0.57 (0.44-0.75)	

Abbreviation: AJCC, American Joint Committee on Cancer.
*Model A was obtained by step-down selection of all prognostic factors (see Supplemental Data 3) and by successive rejection of nonsignificant ($P > 0.10$) variables (with the exception of microsatellite status). In model B, AJCC stage was arbitrarily excluded. HR < 1.00 represents a decreased risk of death, whereas HR > 1.00 represents an increased risk of death.

MSS localized colorectal cancer. Our study was not designed, nor was it powered, for evaluating the effects of 5-FU therapy according to the tumor microsatellite status, but 5-FU treatment was included in the multivariate models of survival (Table 4). This argues against the possibility that MSI has not been recognized as a stage-independent predictor of survival because of a more aggressive use of 5-FU adjuvant therapy. We rather believe that the enforcement of current recommendations for sampling and pathologic assessment of lymph nodes (a mean of more than 20 nodes analyzed in patients with N_0 tumors; see footnote to Table 1), together with the routine use of newer imaging techniques, has provided a more accurate clinico-pathologic staging of the disease. Consistently, our series had a lower prevalence of non-metastatic cancer (43% versus 52% in ref. 18 and versus 48% in ref. 20) and a much higher prevalence of stage IV patients (27% versus 18% in ref. 18 and versus 14% in ref. 20). If metastases remained undetected at initial diagnosis, this more likely occurred among MSS patients who do have a much greater likelihood of metastases. Therefore, the previous recognition of MSI as a stage-independent predictor of survival might simply reflect an inadvertent down-staging of MSS cancers.

Once the lower metastatic risk of MSI cancers was established to be the main determinant of their prognostic advantage, we wanted to see whether the prevalence of metastases was different for molecular subsets of unstable tumor. Confirming previous data (17, 26), we found no difference in lymph node or distant organ metastases and in survival between MSS cancers and MSI tumors with wild-type *TGFβRII*. As entirely novel findings, we observed that MMR protein defect (hMLH1, hMSH2, or hMSH6) and *p16* methylation of sporadic tumors affected the presentation stage of MSI cancers and, in turn, their prognosis.

It has been a long-standing matter of controversy whether survival expectancy of patients with HNPCC should be considered equivalent to that of patients with MSI sporadic colorectal cancer. Benatti et al. recently found a better outcome for HNPCC patients, which was associated with a less advanced tumor stage (20), but no conclusive data are available. The findings of the present study are novel in indicating that only germ-line mutations in *hMSH2* may determine a lower metastatic potential and a better prognosis of HNPCC compared with MSI sporadic cancers.

As to patients with hMLH1-deficient sporadic cancer, only a minority of them (i.e., those with *p16*-unmethylated cancer) had a decreased likelihood of metastases and a prognostic advantage versus patients with MSS cancer. This finding is particularly intriguing because *p16*-promoter methylation is a marker of CpG islands methylation, the molecular phenotype that underlies *hMLH1* methylation and leads to MSI. On the other hand, the association of *p16* methylation with lymph

node metastases from colorectal cancer has been reported, regardless of MSI status (27). Therefore, selective *hMLH1* methylation, which more frequently occurs in cancers from older females (42, 43), seems to identify a subset of MSI cancers with a more favorable prognosis.

In conclusion, the appropriate clinical application of MSI testing in colorectal cancer is to select patients who may carry MMR gene germ-line mutations. Conversely, the assessment of microsatellite status cannot refine the prognostic value of a state-of-the-art clinico-pathologic staging. In particular, the administration of adjuvant chemotherapy should not be withheld by assuming a more favorable outcome in patients with MSI cancer. At the investigational level, MSI testing is warranted in patients entering prospective chemotherapeutic trials to look for possible differential responses, whereas preclinical research should focus on the specific molecular changes, both genetic and epigenetic, that determine the low metastatic potential of MSI cancers.

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