

Adverse Prognostic Effect of Methylation in Colorectal Cancer Is Reversed by Microsatellite Instability

By Robyn Lynne Ward, Kay Cheong, Su-Lyn Ku, Alan Meagher, Terence O'Connor, and Nicholas John Hawkins

Purpose: DNA methylation is an important biologic event in colorectal cancer and in some cases is associated with the development of microsatellite instability (MSI). In this study, we sought to determine the prognostic significance of DNA methylation, both in univariate analysis and in concert with other clinicopathologic factors known to influence outcome.

Patients and Methods: Fresh tissue (625 cancers) was obtained from 605 individuals (age range, 29 to 99 years) undergoing curative surgery for colorectal cancer at one institution during a period of 8 years. Clinicopathologic details were recorded for all tumors, including stage, grade, type, vascular space invasion, and clinical follow-up to 5 years. Microsatellite status was assessed using standard markers. Methylation of *p16* and *hMLH1* promoters was determined by methylation-specific polymerase chain reaction (PCR), whereas methylation at methylated-in-tumor loci (*MINT*)1, *MINT*2, *MINT*12, and *MINT*31 loci were assessed by bisulfite-PCR.

Results: Patients with microsatellite unstable tumors (12%) had better disease-specific survival than those with microsatellite stable (MSS) tumors (univariate analysis: hazard ratio [HR], 0.53; 95% CI, 0.27 to 1.0). Overall survival of individuals with MSS tumors was influenced by three independently significant factors: tumor stage (HR, 7.3; 95% CI, 5.1 to 10.4), heavy tumor methylation (HR, 2.1; 95% CI, 1.1 to 4.0), and vascular space invasion (HR, 1.9; 95% CI, 1.3 to 2.9). In MSS tumors, methylation at any single site was not independently predictive of survival. Neither methylation nor microsatellite status predicted a favorable response to chemotherapy.

Conclusion: DNA methylation is associated with a worse outcome in colorectal cancer, but this adverse prognostic influence is lost in those methylated tumors showing MSI. The mechanisms of these events warrant additional investigation.

J Clin Oncol 21:3729-3736. © 2003 by American Society of Clinical Oncology.

THE CLINICOPATHOLOGIC staging of colorectal carcinoma currently is based on a combination of the extent of local tumor invasion, lymph node status, and the presence or absence of distant metastasis.^{1,2} This staging system provides an excellent indication of prognosis and identifies those likely to benefit from adjuvant therapy. Although stage remains the benchmark, there are a number of other pathologic factors known to provide additional prognostic information. Among these are the extent of residual tumor (R classification),^{3,4} the histologic grade of the tumor, and the presence of vascular space invasion.² A large number of studies using multivariate analysis have unequivocally demonstrated that tumors of high grade, as well as those with vascular invasion, have worse outcomes independent of tumor stage (reviewed by Compton et al²). Despite this evidence, these pathologic factors are not universally reported, largely because of technical and interpretive problems.

During the last two decades, many of the genetic and epigenetic changes that underpin the development of colorectal cancer have been identified. However, the hope that they would be integrated with the routine staging systems has not been realized. For instance, a recent meta-analysis showed that *p53* status was of marginal prognostic value and that its use in clinical practice could not be justified.⁵ Similar conclusions have been drawn from studies of *K-ras* mutations,⁶ 18q loss,^{7,8} and overexpression of *HER-2*⁹ and *c-myc*¹⁰ (reviewed by Pasche et al¹¹).

In contrast to this trend, microsatellite instability (MSI) has shown promise as a prognostic marker, at least in select subgroups of patients, such as those with hereditary nonpolyposis colorectal cancer,¹² in younger individuals,¹³ and in individuals with stage II or III disease.^{14,15} In sporadic colorectal

cancers, microsatellite instability arises through methylation of the *hMLH1* promoter,¹⁶⁻¹⁸ whereas in hereditary nonpolyposis colorectal cancer it occurs through germline mutations of one of the mismatch repair genes.¹⁹ The survival advantage accruing in patients with microsatellite unstable tumors seems to occur regardless of the specific mismatch repair gene involved or the mechanism by which it is inactivated.

It recently has been shown that the subset of sporadic cancers characterized by *hMLH1* methylation and MSI overlaps with a broader group of tumors that display extensive methylation of CpG islands at methylated-in-tumor (*MINT*) loci and *p16*.^{20,21} This larger group of sporadic colorectal cancers, often referred to as CpG island methylator phenotype-positive tumors, shares many of the clinicopathologic features of microsatellite unstable cancers, including right-sided tumor location and mucinous phenotype.²¹⁻²³ However, more than half of the tumors displaying widespread CpG island methylation are microsatellite stable (MSS).²³ It is not known whether these

From the Departments of Medical Oncology and Colorectal Surgery, St Vincent's Hospital, Darlinghurst; and the Schools of Medicine and Medical Sciences, University of New South Wales, Sydney, Australia.

Submitted March 19, 2003; accepted July 30, 2003.

Authors' disclosures of potential conflicts of interest are found at the end of this article.

Address reprint requests to Robyn Ward, MD, Department of Medical Oncology, St Vincent's Hospital, Victoria St, Darlinghurst, NSW 2010, Australia; e-mail: r.ward@garvan.unsw.edu.au.

© 2003 by American Society of Clinical Oncology.

0732-183X/03/2120-3729/\$20.00

Table 1. Patient Characteristics

Characteristic	Colon		Rectum	
	No.	%	No.	%
No. of patients	385		236	
Age, years		69.7		66.0
Standard deviation		12.0		12.2
Range		31-99		29-99
Sex				
Female		198		84
Male		179		144
No. of tumors		393		232
Tumor stage				
I	65	16.4	56	24.1
II	154	39.0	70	30.2
III	113	29.7	78	34.5
IV	61	14.5	28	11.3
Adjuvant chemotherapy†				
I	0 of 59	0	1 of 53	1.8
II	24 of 108	22	14 of 70	20
III	79 of 111	71	54 of 77	70
Radiotherapy‡				
I		0		2 preoperation
II		1 preoperation		7 preoperation, 8 postoperation
III		2 postoperation		9 preoperation, 24 postoperation
IV		0		4 preoperation, 6 postoperation
Microsatellite status				
Stable	319	81	228	98
Unstable	74	19	4	2

*Total of 605 patients enrolled, eight with cancers arising synchronously in colon and rectum.

†Percentage of patients receiving treatment according to stage shown. Regimens used were Roswell Park (52%), Mayo (39%), Moertel (3.4%), and other (5.7%). Four patients with stage II cancers and 11 with stage III cancers did not complete a full course of adjuvant chemotherapy.

‡Forty-three patients received infusional fluorouracil during radiotherapy.

MSS methylated tumors share the improved prognosis of their microsatellite unstable cousins.

In this study, we evaluated the prognostic significance of methylation and microsatellite status in 605 consecutive colorectal cancer patients. We also undertook an extensive pathologic assessment of each tumor to establish the importance of these molecular changes in relation to standard clinicopathologic criteria.

PATIENTS AND METHODS

Patients and Specimens

After providing informed consent, 605 consecutive individuals (with 625 tumors) undergoing complete (RO or R1)¹ surgical resection of colorectal cancer at St Vincent's Hospital (Darlinghurst, Australia) were entered into this prospective study. This study was approved by the Hospital Ethics Committee. Enrollment was from January 1, 1994 to August 1, 2002, and patients were excluded if they had inflammatory bowel disease or a known hereditary colorectal cancer.

Treatment with chemotherapy and/or radiotherapy was recorded (Table 1), and treatment decisions were made without knowledge of the genetic characteristics of the tumor. In all patients, adjuvant therapy was one of three standard fluorouracil-based regimens.²⁴⁻²⁶ External-beam radiotherapy, with or without radiosensitization with fluorouracil, was administered according to standard protocols.²⁷ Palliative chemotherapy was administered at the discretion of the treating physician, and response was not recorded. Vital status of study participants was obtained as of September 15, 2002, by which time 10 (1.6%) of 605 individuals were

lost to follow-up. The follow-up was undertaken for a period of 5 years or until death. Cancer recurrence dates and causes of death were obtained from death certificates or from the medical record.

Collection and Histopathologic Analysis of Tumors

Fresh representative tissue samples (500 µg) from all tumors and paired normal colonic mucosa were immediately frozen at -70°C. Of the 625 colorectal carcinomas collected, 121 (19.3%) were tumor-node-metastasis system stage I, 224 (35.7%) were stage II, 191 (31%) were stage III, and 89 (14%) were stage IV.¹ There were 27 individuals with a total of 47 synchronous tumors. In 16 of these individuals, fresh tumor was collected from all lesions (36 tumors), whereas only the most advanced lesion was collected in the remaining 11 patients. Histopathologic assessment of all tumors was performed as previously described.²³ Vascular space invasion was defined as the presence of viable tumor cells wholly within an endothelial-lined space or structure, as recognized by light microscopy.

Microsatellite Status

The microsatellite status of each tumor was determined as previously described, using the following primer sets: Bat 25, Bat 26, Bat 40, D5S346, D2S123, and D17S250.²⁸ Tumors with instability at two or more markers were considered microsatellite unstable, whereas all others were designated as MSS.

Detection of Methylation of p16, hMLH1, MINT1, MINT2, MINT12, and MINT31

For methylation analyses, DNA was extracted and treated with sodium bisulfite as previously described.²³ Methylation-specific polymerase chain

reaction was performed to detect methylation of the *p16* promoter region.^{23,29} All amplicons generated by the methylation-specific *p16* primers were digested with 20 U of *BstU* 1 at 60°C overnight (New England Biolabs Inc, Beverly, MA) to confirm methylated DNA, given that the methylated amplicon has two restriction sites for this enzyme, whereas the unmethylated amplicon has none. The methylation status of *MINT1*, *MINT2*, *MINT12*, and *MINT31* was determined according to the method of Toyota et al.²¹ with modifications as previously described.²³ Methylation of *hMLH1* was examined as previously described,³⁰ using bisulfite–restriction fragment length polymorphism at two separate regions of the *hMLH1* promoter (A and C) reported to be associated with *hMLH1* silencing.^{17,31} Amplicons from methylated or unmethylated template were distinguished by restriction enzyme digestion with 20 U of *BstU* 1.

For *hMLH1*, *p16*, and each of the *MINT* loci, methylation status was recorded as either unmethylated, methylated, or not assessable. Positive and negative controls^{23,30} were included for each reaction. The methylation status of each tumor was reported as the number of sites methylated from a total of five (four *MINT* loci and *p16*), and was not assessable in 57 (9%) tumors. A tumor was defined as heavily methylated when more than three of five loci were methylated.^{21–23}

Statistical Analysis

Categorical variables were compared using the χ^2 test or the Fisher's exact test. For individuals with synchronous tumors, the highest stage lesion alone was used in survival analysis. Survival was measured from the date of resection of colorectal cancer to the date of death, the completion of 5 years of follow-up, or the last clinical review before September 1, 2002. Only cancer-related deaths were analyzed as events. Time to recurrence was the interval from surgery to documented tumor recurrence. Differences between Kaplan-Meier survival curves were tested using the log-rank test. Univariate survival analysis was performed by Cox proportional hazards regression model, and multivariate analysis was used to determine independent prognostic factors. A *P* value of less than .05 was considered significant. All data were analyzed using SPSS statistical software, Version 11.0 (SPSS Inc, Chicago, IL).

RESULTS

Patient Characteristics

The study population (Table 1) consisted of 282 females and 323 males, with a mean age of 68.3 ± 12.2 years (range, 29 to 99 years). At the time of resection, 13% of individuals were younger than 55 years, 54% were between 55 and 74 years, and 33% were older than 75 years. After surgical resection of the primary cancer, 53 individuals with rectal cancer were treated with either preoperative or postoperative radiotherapy, whereas 171 (42%) of the individuals with stage II or III cancer received adjuvant chemotherapy. Complete surgical resection of metastases was performed in three patients who had presented with a stage IV tumor, and in an additional eight individuals subsequent to hepatic recurrence. At the censor date of September 1, 2002, 434 patients were alive, 129 had died as a result of cancer, and 42 had died as a result of unrelated causes. The median follow-up period was 32 months (range, 1 to 60 months), with 144 individuals having more than 4 years of follow-up. Disease recurred subsequent to resection in 81 individuals, with a median time to recurrence of 16.6 months (range, 3.2 to 58.8 months).

CpG Island Methylation at Specific Loci

Table 2 shows the relationships between the presence of methylation at individual loci and a range of clinicopathologic

features of individuals and their tumors. As noted in previous studies,^{21–23} methylation at each locus was more common in older individuals and in females. Furthermore, irrespective of the locus analyzed, methylation was associated with right-sidedness, mucinous tumor type, prominent intraepithelial lymphocytes, and MSI (Table 2). There was no association between methylation and the presence of tumor in either the vascular or the lymphatic space.

Prognostic Factors for Survival

As expected, advanced tumor stage and the presence of vascular space invasion were associated with significantly decreased survival for all patients, as well as for those who had undergone curative resections for nonmetastatic disease (stages I to III; Table 3). When all tumor stages were considered, patients with microsatellite unstable tumors had better disease-specific survival than patients with MSS tumors (Table 3). Only nine of 73 (12%) individuals with microsatellite unstable cancers had died within the study period, compared with 120 of 532 individuals (23%) with MSS tumors. At first examination, it seemed that the degree of tumor methylation did not significantly affect disease outcome (Table 3). However, this analysis was confounded by the finding that MSI, which represented the majority of methylated cancers, correlated with both improved survival and with widespread CpG island methylation ($P < .0001$; Table 4). Examination of survival curves demonstrated that increasing levels of tumor methylation were not related to outcome for the microsatellite unstable tumors but were significantly associated with decreased survival within the MSS cancer group (Fig 1). This latter trend was most apparent with tumors showing methylation at more than three loci but was also noted in tumors with more than two methylated sites. Given the relationship between microsatellite status and methylation, we elected to divide tumors into stable or unstable groups before survival analysis (Fig 2).

For individuals with MSS tumors, Cox's univariate analysis showed that tumor stage, vascular space invasion, poor differentiation, and tumor methylation were of prognostic significance overall (stages I to IV), as well as in nonmetastatic tumors (stages I to III). The final Cox regression model identified three of these as independent prognostic factors: tumor stage, tumor methylation, and vascular space invasion (Table 5).

Methylation of the *MINT* loci and *p16* are closely correlated, and it was considered possible that the adverse effects of methylation of one member of the group could be incorrectly ascribed to tumors displaying a generalized methylator phenotype. To examine this possibility, the prognostic significance of each marker was independently examined. Methylation of *MINT1*, *MINT2*, and *p16* were unrelated to disease outcome, with hazard ratios (HRs) of 1.1 (95% CI, 0.73 to 1.9), 1.5 (95% CI, 1.0 to 2.3), and 1.4 (95% CI, 0.93 to 2.2), respectively. In univariate analysis, it was evident that methylation of *MINT12* and *MINT31* was associated with poor prognosis (HR, 1.7; 95% CI, 1.2 to 2.3; $P = .01$ and HR, 2.0; 95% CI, 1.2 to 3.5; $P = .02$, respectively). However, methylation at either of these loci was not independently significant when analyzed with stage, vascular space invasion, and grade.

Table 2. Patient Characteristics and Phenotypic Features of Cancers Showing CpG Island Methylation at Specific Loci

	MINT1		MINT2		MINT2		MINT31		p16											
	M	UM	M	UM	M	UM	M	UM	M	UM										
	No.	%	No.	%	No.	%	No.	%	No.	%										
Patients	125	23	428	77	150	29	362	71	141	26	407	74	77	14	468	86	128	23	427	77
Age, years																				
Mean	71.5		67.4		71.6		66.9		70.8		67.4		72.6		67.6		72.1		67.3	
SD	11.7		12.1		12.5		12.1		12.2		12.1		11.0		12.3		11.9		12.1	
P			.001				.003				.004				.001				< .001	
Sex																				
Female	13		34		15		31		14		32		7		39		14		32	
Male	10		43		14		40		12		42		7		47		9		45	
P			.02				.07				.01				.2				< .001	
Tumors	128	23	437	77	155	30	368	70	143	25	417	75	79	14	478	86	131	23	436	77
Side																				
Right	13		24		16		21		13		23		9		28		15		22	
Left	9		54		13		50		12		52		5		58		8		55	
P			< .001				< .001				< .001				< .001				< .001	
Grade																				
High	6		9		7		8		4		11		4		11		7		9	
Low	16		69		23		62		22		63		11		74		16		68	
P			< .001				.003				NS				.006				< .001	
Mucinous																				
Yes	8		9		10		10		7		12		6		13		7		12	
No	16		67		20		60		18		63		8		73		16		65	
P			< .001				< .001				.001				< .001				< .001	
IEL																				
Increased	9		10		8		11		8		12		5		14		9		11	
Normal	14		67		21		60		17		63		9		72		14		66	
P			< .001				.001				< .001				< .001				< .001	
MSI																				
Present	9		5		9		4		7		6		6		7		7		7	
Absent	14		72		20		67		18		69		8		79		16		70	
P			< .001				< .001				< .001				< .001				< .001	

Abbreviations: MINT, methylated-in-tumor gene; M, methylated; UM, unmethylated; SD, standard deviation; IEL, intraepithelial lymphocytes; MSI, microsatellite instability.

With regard to individuals with microsatellite unstable cancers, tumor stage (HR, 6.2; 95% CI, 2.4 to 16.2; $P < .0001$), right-sided location (HR, 0.13; 95% CI, 0.03 to 0.48; $P = .002$), and age (HR, 1.0; 95% CI, 1.0 to 1.2; $P = .03$) were the only

factors that correlated with disease outcome. In fact, five of the nine individuals who died as a result of a microsatellite unstable cancer had stage IV disease at presentation, and the mean age of the deceased group (80.5 ± 7.9 years) was significantly older

Table 3. Pathologic and Genetic Parameters Influencing Survival

Factor	No. of Patients	Stages I to IV			Stages I to III			P	Recurrence-Free Survival		
		Overall Survival			Overall Survival				Recurrence-Free Survival		
		HR	95% CI	PS	HR	95% CI	P		HR	95% CI	P
Tumor stage	605	8.0	5.9 to 10.7	< .0001	516	4.5	2.7 to 7.5	< .0001	3.3	2.2 to 4.7	< .0001
MSI	605	0.52	0.27 to 1.0	.06	516	0.5	0.17 to 1.3	.15	0.5	0.1 to 1.1	.10
Heavy methylation	556	1.3	0.7 to 2.2	.37	476	1.2	0.6 to 2.8	.60	0.9	0.5 to 2.0	.94
Vessel positive*	605	4.6	3.2 to 6.6	< .0001	516	3.7	2.2 to 6.2	< .0001	3.3	2.2 to 5.1	< .0001
Margin positive†	605	4.7	3.3 to 6.5	< .0001	516	2.2	0.7 to 7.0	.19	2.0	0.7 to 5.4	.18
Sex, male	605	1.3	0.94 to 1.9	.10	516	1.5	0.9 to 2.5	.16	1.6	0.99 to 2.5	.05
High grade	605	2.1	1.4 to 3.1	.001	516	1.6	0.83 to 3.2	.16	1.5	0.8 to 2.8	.17
Right-sided location‡	605	0.86	0.6 to 1.2	.43	516	0.74	0.4 to 1.3	.30	0.7	0.4 to 1.1	.12

NOTE. In patients with synchronous tumors, only the properties of the most advanced tumor were considered in this analysis. Heavily methylated tumors were defined as those methylated at more than three of five sites. Methylation status was not assessable in 49 patients.

Abbreviations: HR, hazard ratio; MSI, microsatellite instability.

*Presence of vascular space invasion.

†Presence of microscopic residual disease at resection margins (tumor-node-metastasis system R1).

‡Tumor proximal to splenic flexure.

§P values < .05 considered significant; P trend reported for methylation status.

Table 4. Characteristics of Tumors According to Microsatellite Status

	With MSI		MSS	
	No.	%	No.	%
No. of tumors	78		547	
Site				
Right colon	65	83	168	31
Left colon	9	11	151	28
Rectum	4	5	228	42
Tumor stage				
I	15	12	106	19
II	42	41	182	33
III	15	14	176	32
IV	6	6	83	15
Mucinous				
Yes	35	47	89	16
No	43	53	458	84
Vessel invasion				
Yes	19	26	190	35
No	59	74	357	65
Sites methylated*				
0	12	15.5	249	51
1	10	14.1	141	29
2	7	8.5	49	10
3	12	14.1	30	6
4	15	21.1	17	3.5
5	19	26.8	7	1.4

NOTE. In this study, 78 tumors (12.8%) were microsatellite unstable; they were more likely to be right-sided, mucinous, and methylated. Of the 58 heavily methylated tumors (methylated at more than three of five sites), 34 had MSI.

Abbreviations: MSI, microsatellite instability; MSS, microsatellite stable.

*Sites assessed were the four methylated in tumor (*MINT*) gene loci and *p16*.

than that of the remaining patients (mean, 70.5 ± 13.6 years; $P = .006$). Interestingly, vascular space invasion and mucinous phenotype showed no relationship to survival.

Analysis of methylation at two regions of the *hMLH1* promoter was performed in a subset of tumors ($n = 383$) in this study. Methylation of both regions of the promoter was strongly associated with MSI and with methylation of the five other loci examined ($P < .0001$). Eighty percent of the assessable microsatellite unstable tumors displayed methylation of region C, 83% were methylated at region A, and 70% of tumors showed methylation at both regions. There was no association between methylation of either region and survival. Methylation was found less frequently in assessable MSS tumors, with 11% methylated at region A, 23% at region C, and 2.4% at both regions. In individuals with MSS tumors, we noted a

trend toward reduced survival in patients with methylation at either region, but this difference was not significant (HR, 1.6; 95% CI, 0.9 to 2.6; $P = .09$).

Factors Influencing Response to Chemotherapy

A total of 133 (70%) of 188 stage III and 38 (21%) of 178 stage II individuals received chemotherapy after curative resection of their primary tumor. Predictably, these individuals were significantly younger (mean age, 64 ± 11 years; $P < .0001$) than their untreated counterparts of similar stage (mean age, 72 ± 12 years). Stage for stage, there were no significant differences between treated and untreated groups in terms of sex, proportion of right-sided tumors, or frequency of vascular space invasion. Adjuvant therapy was associated with a significant reduction in risk of death for stage III patients overall (HR, 0.2; 95% CI, 0.12 to 0.4; $P < .0001$). This difference also held true when rectal tumors (HR, 0.22; 95% CI, 0.10 to 0.5; $P < .0001$) and colonic tumors (HR, 0.22; 95% CI, 0.10 to 0.5; $P < .0001$) were considered separately. With regard to stage II tumors, there was no overall difference in survival between those receiving chemotherapy and those in whom the treatment was not administered. When the subgroup of individuals who received chemotherapy on an intent-to-treat basis was considered, neither the extent of methylation nor any other clinicopathologic factor assessed in this study predicted a response to treatment (Table 6).

DISCUSSION

In this study, we have used a prospective collection of 625 colorectal cancers to examine the prognostic significance of methylation and MSI. There are a number of well-established pathologic criteria that readily identify prognostic subgroups of colorectal cancer. These criteria were determined in this study to establish a framework against which new molecular information could be assessed. A strength of this study is that it was broadly representative of standard clinical practice. For instance, more than 30% of patients were older than 75 years, a group that is often excluded when studies are performed using material collected in the course of a clinical drug trial.

A key finding of this study was that individuals with heavily methylated but MSS tumors had a significantly worse outcome than those with nonmethylated MSS tumors. One explanation for this observation is that widespread methylation is simply a surrogate marker for some other well-established adverse patho-

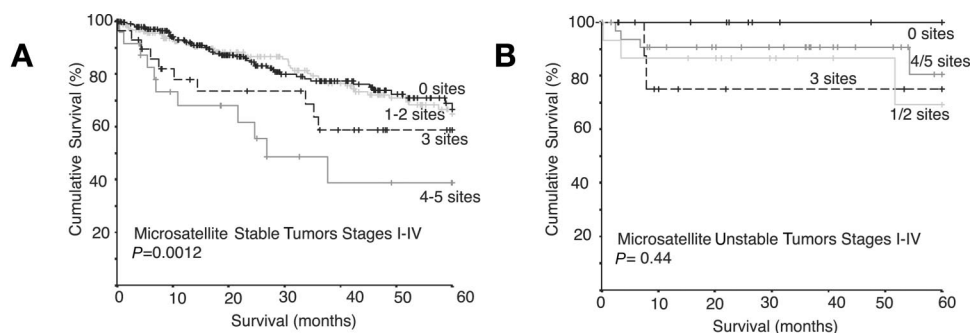


Fig 1. Kaplan and Meier plot showing the effects of increasing methylation on survival for (A) microsatellite stable (MSS) and (B) microsatellite unstable tumors. MSS tumors with widespread CpG island methylation (four to five sites) show decreased survival compared to tumors with minimal methylation (one to two sites) or no methylation (no sites).

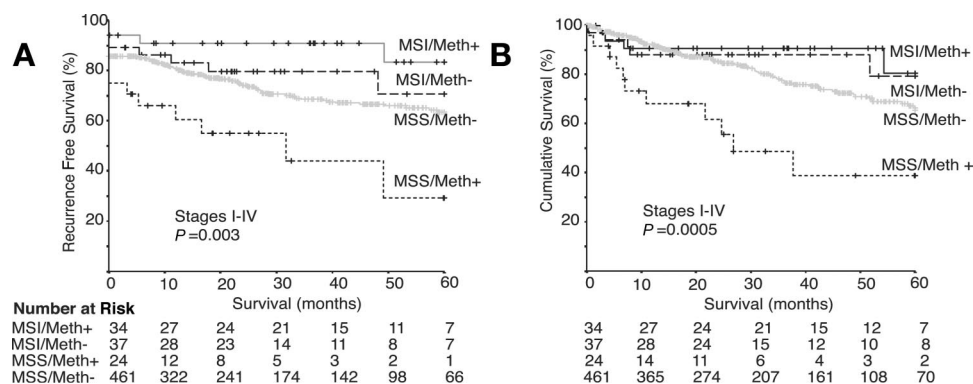


Fig 2. (A) Recurrence-free survival and (B) overall survival of individuals with stages I to IV cancers according to methylation and microsatellite status. Tumors categorized as either microsatellite unstable (MSI) or microsatellite stable (MSS) with or without heavy methylation. Meth⁺, heavy methylation (methylation at > 3 of 5 sites).

logic characteristic. Although this possibility cannot be completely excluded in this study, it seems unlikely, given that the prognostic significance of methylation was shown to be independent of powerful predictors of outcome, such as tumor stage and vascular space invasion.

A second possibility is that the presence of methylation obviates the positive effects of adjuvant chemotherapy. Although there are no published data concerning the interactions between CpG island methylation and sensitivity to fluorouracil, methylation of the DNA repair enzyme O(6)-methylguanine DNA methyltransferase has been shown to predict the response of gliomas to alkylating agents.³² Our study was not specifically designed to assess interactions between chemosensitivity and molecular changes. Nevertheless, we found no evidence that tumor methylation was related to disease response in those individuals receiving chemotherapy.

It is also possible that methylation, by altering expression of a key gene or group of genes, has altered the biology of the tumor cells in ways that lead to a worse clinical outcome. It is well established that dense hypermethylation of promoter regions can reduce or silence gene expression. In genes such as *hMLH1*, this is strongly linked to the development of MSI colorectal cancers, which are known to have a less aggressive clinical course.^{17,33} Hypermethylation of other genes, such as *BRCA1*,³⁴ E-cadherin,³⁵ *p16*,³⁶ *MGMT*,³⁷ and *p14*^{38,39} have also been described in a variety of tumors, including colorectal cancer. Although these genes have well-defined cellular functions, their methylation does not necessarily alter gene expression,^{40,41} and thus influence tumor outcome. Our study included three genes with potentially important cellular functions, namely *p16*, *hMLH1*, and

MINT31.⁴² However, we found no association between methylation at *p16* or *hMLH1* and changes in survival or disease-free recurrence. For *MINT31*, there was a weak association between methylation and survival in univariate analysis, but this was not an independent prognostic factor. It is thus unlikely that methylation of any one of the individual genes examined in this study was responsible for the poor outcome observed with methylated tumors. Clearly, we have not eliminated the possibility that methylation of one or more other critical genes has occurred and that this process is indirectly associated with methylation of our gene panel.

Apart from speculating on its possible causes, the decreased survival seen in methylated MSS cancer group is of interest for at least one other reason. It has recently been suggested that although the extent of methylation is distributed widely among cancers, those tumors with the greatest degree of methylation also show preferential methylation of particular groups of CpG islands.⁴³ Thus the assignment of some colorectal cancers into a hypermethylator group may reflect qualitative as well as quantitative changes in DNA methylation. Certainly, the finding of this study that heavily methylated cancers have a distinctive clinical course tends to support the biologic validity of this grouping.

The second key finding of this study relates to prognosis in microsatellite unstable cancers. Approximately 12% of the tumors in this study demonstrated MSI, and in univariate analysis, these tumors were associated with a modest improvement in overall survival that did not reach statistical significance. In a study of 587 individuals younger than 50 years of age, MSI has previously been found to be an independent prognostic

Table 5. Significant Prognostic Factors in Microsatellite Stable Cancers

Risk Factor	Stages I-IV (N = 532)						Stages I-III (N = 449)					
	Univariate			Multivariate			Univariate			Multivariate		
	HR	95% CI	P	HR	95% CI	P	HR	95% CI	P	HR	95% CI	P
Stage	8.0	5.9 to 10.9	< .0001	7.3	5.1 to 10.4	< .001	4.7	2.6 to 8.3	< .001	4.0	2.2 to 7.2	< .001
Heavy methylation*	3.0	1.6 to 5.6	.001	2.1	1.1 to 4.0	.02	3.3	1.3 to 8.3	.01	2.4	0.94 to 6.0	.06
Vascular space invasion	4.7	3.3 to 6.9	< .0001	1.9	1.3 to 2.9	.002	3.5	2.0 to 6.0	< .0001	2.4	1.4 to 4.2	.003
High tumor grade	2.5	1.6 to 4.0	< .0001	1.6	0.9 to 2.6	.08	1.9	0.98 to 4.2	.09		NA	

NOTE. For pathologic variables, n = total number in the analysis. In the analysis of methylation, 484 and 410 tumors were assessable for stages I to IV and I to III, respectively.

Abbreviations: HR, hazard ratio; NA, not assessable.

*Heavily methylated tumors defined as those methylated at more than three of five sites.

Table 6. Prognostic Factors for Patients Receiving Adjuvant Chemotherapy

Factor	No. of Patients	HR	95% CI	P
Stage*				
II	38	3.2	0.76 to 14.0	.11
III	134			
Sex				
Female	72	0.55	0.2 to 1.4	.21
Male	99			
Location				
Right side	51	0.47	0.2 to 1.4	.18
Left side	120			
Heavy methylation†				
No	149			
Yes	15	1.8	0.24 to 13.6	.56

NOTE. Risk factors were female sex, right-sided location, and heavy methylation. Abbreviation: HR, hazard ratio.

*One patient with a stage I rectal cancer was excluded from the analysis.

†Heavy methylation is methylation at more than three of five sites.

factor.¹³ Likewise, others have found MSI to be an important prognostic factor in specific stages of tumor.¹⁴ However, several studies have detected only a weak survival advantage^{44,45} or no significant survival advantage.⁴⁶⁻⁴⁸

As expected, many of the microsatellite unstable tumors displayed methylation of *hMLH1*, *MINT* loci, or *p16*,^{21,23} yet in striking contrast to MSS tumors, the extent or even presence of methylation in these tumors had no impact on outcome. It has been proposed that methylated microsatellite unstable and MSS tumors arise from similar precursor lesions that are susceptible to a process of CpG island methylation.⁴⁹ Furthermore, there are striking similarities between tumors showing CpG island methylation, irrespective of their microsatellite status.²³ Such tumors have a propensity to develop on the right side of the colon in older women and often display a mucinous phenotype. If this is the case, then it seems that the development of MSI may act as an antidote to the adverse prognostic effects of widespread methylation. Indeed, the apparently minor survival gains associated with the MSI phenotype become considerably more impressive when related to the outcome of methylated MSS cancers.

The mechanisms underlying the improved survival seen in microsatellite unstable cancers remain unknown. It is possible they relate directly to instability, with MSI presumably making cells less fit to progress, or at least to metastasize. This is somewhat paradoxical,⁵⁰ given that instability, either microsat-

ellite or chromosomal in nature, is often invoked as a mechanism driving tumor progression. An alternate hypothesis for improved survival in microsatellite unstable tumors relates to immune mechanisms. Microsatellite unstable tumors typically display an intense intraepithelial infiltrate with CD8⁺CD103⁺ lymphocytes, and it is possible that these T cells play a role in modulating disease progression.⁵¹ In light of these data, it is interesting to note that the intraepithelial and peritumoral lymphocytic infiltrate typical of microsatellite unstable tumors is less common in MSS tumors with heavy methylation.^{23,52}

The clinical implications of the findings of this study remain uncertain. It is premature to suggest that methylation or microsatellite DNA analysis should form part of the standard assessment of all colorectal cancers. These techniques cannot be justified in routine practice on the basis of either economics or clinical need. However, methylated tumors, both stable and unstable, have relatively distinct clinicopathologic features that can be recognized by pathologists and clinicians alike.⁵³ Immunoperoxidase staining for the mismatch repair proteins may supplement standard histopathologic assessment of tumors to support a clinical suspicion of microsatellite instability. The important practical implication of this study is, however, that there is a close pathologic similarity between microsatellite unstable tumors and methylated MSS lesions. Given the significantly different prognosis for these two groups of cancers, it is important that clinicians and pathologists be aware of this distinction.

In conclusion, this study shows that methylation status is an independent prognostic factor in colorectal carcinoma and highlights two important mechanistic questions: why do methylated MSS cancers behave so poorly, and how does the development of microsatellite instability so powerfully reverse this behavior? The answers to these questions may be of considerable relevance to the biology and therapeutics of colorectal cancer.

ACKNOWLEDGMENT

The authors acknowledge Rachael Williams for her assistance in the collection and verification of family history and survival data; Emma Quinn for microsatellite assays; Matthew Law, PhD, for advice on statistics; and Jenny Turner, MD, for review of histopathologic specimens.

AUTHORS' DISCLOSURES OF POTENTIAL CONFLICTS OF INTEREST

The authors indicated no potential conflicts of interest.

REFERENCES

- Hermanek P, Sobin LH: TNM: American Joint Committee on Cancer—Manual for Staging of Cancer. Philadelphia, PA, Springer Verlag, 1998
- Compton C, Fenoglio-Preiser CM, Pettigrew N, et al: American Joint Committee on Cancer Prognostic Factors Consensus Conference: Colorectal Working Group. *Cancer* 88:1739-1757, 2000
- Hermanek P, Wittekind C: Residual tumor (R) classification and prognosis. *Semin Surg Oncol* 10:12-20, 1994
- Newland RC, Dent OF, Chapuis PH, et al: Clinicopathologically diagnosed residual tumor after resection for colorectal cancer: A 20-year prospective study. *Cancer* 72:1536-1542, 1993
- Petersen S, Thames HD, Nieder C, et al: The results of colorectal cancer treatment by p53 status: Treatment-specific overview. *Dis Colon Rectum* 44:322-333, 2001
- Andreyev HJ, Norman AR, Cunningham D, et al: Kirsten ras mutations in patients with colorectal cancer: The "RASCAL II" study. *Br J Cancer* 85:692-696, 2001
- Jen J, Kim H, Piantadosi S, et al: Allelic loss of chromosome 18q and prognosis in colorectal cancer. *N Engl J Med* 331:213-221, 1994
- Dix BR, Robbins P, Soong R, et al: The common molecular genetic alterations in Dukes' B and C colorectal carcinomas are not short-term prognostic indicators of survival. *Int J Cancer* 59:747-751, 1994
- Sun XF, Carstensen JM, Stal O, et al: c-erbB-2 oncoprotein in relation to DNA ploidy and prognosis in colorectal adenocarcinoma. *APMIS* 103:309-315, 1995
- Augenlicht LH, Wadler S, Corner G, et al: Low-level c-myc amplification in human colonic carcinoma cell lines and tumors: A frequent,

p53-independent mutation associated with improved outcome in a randomized multi-institutional trial. *Cancer Res* 57:1769-1775, 1997

11. Pasche B, Mulcahy M, Benson AB III: Molecular markers in prognosis of colorectal cancer and prediction of response to treatment. *Best Pract Res Clin Gastroenterol* 16:331-345, 2002

12. Watson P, Lin KM, Rodriguez-Bigas MA, et al: Colorectal carcinoma survival among hereditary nonpolyposis colorectal carcinoma family members. *Cancer* 83:259-266, 1998

13. Gryfe R, Kim H, Hsieh ET, et al: Tumor microsatellite instability and clinical outcome in young patients with colorectal cancer. *N Engl J Med* 342:69-77, 2000

14. Wright CM, Dent OF, Barker M, et al: Prognostic significance of extensive microsatellite instability in sporadic clinicopathological stage C colorectal cancer. *Br J Surg* 87:1197-1202, 2000

15. Elsaleh H, Powell B, McCaul K, et al: P53 alteration and microsatellite instability have predictive value for survival benefit from chemotherapy in stage III colorectal carcinoma. *Clin Cancer Res* 7:1343-1349, 2001

16. Veigl ML, Kasturi L, Olechnowicz J, et al: Biallelic inactivation of hMLH1 by epigenetic gene silencing, a novel mechanism causing human MSI cancers. *Proc Natl Acad Sci U S A* 95:8698-8702, 1998

17. Herman JG, Umar A, Polyak K, et al: Incidence and functional consequences of hMLH1 promoter hypermethylation in colorectal carcinoma. *Proc Natl Acad Sci U S A* 95:6870-6875, 1998

18. Furukawa T, Konishi F, Masubuchi S, et al: Densely methylated MLH1 promoter correlates with decreased mRNA expression in sporadic colorectal cancers. *Genes Chromosomes Cancer* 35:1-10, 2002

19. Menigatti M, Di Gregorio C, Borghi F, et al: Methylation pattern of different regions of the MLH1 promoter and silencing of gene expression in hereditary and sporadic colorectal cancer. *Genes Chromosomes Cancer* 31:357-361, 2001

20. Ahuja N, Mohan AL, Li Q, et al: Association between CpG island methylation and microsatellite instability in colorectal cancer. *Cancer Res* 57:3370-3374, 1997

21. Toyota M, Ahuja N, Ohe-Toyota M, et al: CpG island methylator phenotype in colorectal cancer. *Proc Natl Acad Sci U S A* 96:8681-8686, 1999

22. Toyota M, Ohe-Toyota M, Ahuja N, et al: Distinct genetic profiles in colorectal tumors with or without the CpG island methylator phenotype. *Proc Natl Acad Sci U S A* 97:710-715, 2000

23. Hawkins N, Norrie M, Cheong K, et al: CpG island methylation in sporadic colorectal cancers and its relationship to microsatellite instability. *Gastroenterology* 122:1376-1387, 2002

24. Moertel CG, Fleming TR, Macdonald JS, et al: Levamisole and fluorouracil for adjuvant therapy of resected colon carcinoma. *N Engl J Med* 322:352-358, 1990

25. O'Connell MJ, Mailliard JA, Kahn MJ, et al: Controlled trial of fluorouracil and low-dose leucovorin given for 6 months as postoperative adjuvant therapy for colon cancer. *J Clin Oncol* 15:246-250, 1997

26. Wolmark N, Rockette H, Fisher B, et al: The benefit of leucovorin-modulated fluorouracil as postoperative adjuvant therapy for primary colon cancer: Results from National Surgical Adjuvant Breast and Bowel Project protocol C-03. *J Clin Oncol* 11:1879-1887, 1993

27. Glimelius B: Radiotherapy in rectal cancer. *Br Med Bull* 64:141-157, 2002

28. Ward R, Meagher A, Tomlinson I, et al: Microsatellite instability and the clinicopathological features of sporadic colorectal cancer. *Gut* 48:821-829, 2001

29. Herman JG, Graff JR, Myohanen S, et al: Methylation-specific PCR: A novel PCR assay for methylation status of CpG islands. *Proc Natl Acad Sci U S A* 93:9821-9826, 1996

30. Suter C, Norrie M, Ku S, et al: CpG island methylation is a common finding in colorectal cancer cell lines. *Br J Cancer* 88:413-419, 2003

31. Deng G, Chen A, Hong J, et al: Methylation of CpG in a small region of the hMLH1 promoter invariably correlates with the absence of gene expression. *Cancer Res* 59:2029-2033, 1999

32. Esteller M, Garcia-Foncillas J, Andion E, et al: Inactivation of the DNA-repair gene MGMT and the clinical response of gliomas to alkylating agents. *N Engl J Med* 343:1350-1354, 2000

33. Kane MF, Loda M, Gaida GM, et al: Methylation of the hMLH1 promoter correlates with lack of expression of hMLH1 in sporadic colon tumors and mismatch repair-defective human tumor cell lines. *Cancer Res* 57:808-811, 1997

34. Esteller M, Silva JM, Dominguez G, et al: Promoter hypermethylation and BRCA1 inactivation in sporadic breast and ovarian tumors. *J Natl Cancer Inst* 92:564-569, 2000

35. Tamura G, Yin J, Wang S, et al: E-cadherin gene promoter hypermethylation in primary human gastric carcinomas. *J Natl Cancer Inst* 92:569-573, 2000

36. Esteller M, Gonzalez S, Riques RA, et al: K-ras and p16 aberrations confer poor prognosis in human colorectal cancer. *J Clin Oncol* 19:299-304, 2001

37. Esteller M, Toyota M, Sanchez-Cespedes M, et al: Inactivation of the DNA repair gene O6-methylguanine-DNA methyltransferase by promoter hypermethylation is associated with G to A mutations in K-ras in colorectal tumorigenesis. *Cancer Res* 60:2368-2371, 2000

38. Zheng S, Chen P, McMillan A, et al: Correlations of partial and extensive methylation at the p14(ARF) locus with reduced mRNA expression in colorectal cancer cell lines and clinicopathological features in primary tumors. *Carcinogenesis* 21:2057-2064, 2000

39. Esteller M, Tortola S, Toyota M, et al: Hypermethylation-associated inactivation of p14(ARF) is independent of p16(INK4a) methylation and p53 mutational status. *Cancer Res* 60:129-133, 2000

40. Fearon ER: BRCA1 and E-cadherin promoter hypermethylation and gene inactivation in cancer-association or mechanism? *J Natl Cancer Inst* 92:515-517, 2000

41. Costello JF, Plass C: Methylation matters. *J Med Genet* 38:285-303, 2001

42. Toyota M, Ho C, Ohe-Toyota M, et al: Inactivation of CACNA1G, a T-type calcium channel gene, by aberrant methylation of its 5' CpG island in human tumors. *Cancer Res* 59:4535-4541, 1999

43. Costello JF, Fruhwald MC, Smiraglia DJ, et al: Aberrant CpG-island methylation has non-random and tumour-type-specific patterns. *Nat Genet* 24:132-138, 2000

44. Halling KC, French AJ, McDonnell SK, et al: Microsatellite instability and 8p allelic imbalance in stage B2 and C colorectal cancers. *J Natl Cancer Inst* 91:1295-1303, 1999

45. Elsaleh H, Joseph D, Griev F, et al: Association of tumour site and sex with survival benefit from adjuvant chemotherapy in colorectal cancer. *Lancet* 355:1745-1750, 2000

46. Johannsdottir JT, Bergthorsson JT, Gretarsdottir S, et al: Replication error in colorectal carcinoma: Association with loss of heterozygosity at mismatch repair loci and clinicopathological variables. *Anticancer Res* 19:1821-1826, 1999

47. Salahshor S, Kressner U, Fischer H, et al: Microsatellite instability in sporadic colorectal cancer is not an independent prognostic factor. *Br J Cancer* 81:190-193, 1999

48. Messerini L, Ciantelli M, Baglioni S, et al: Prognostic significance of microsatellite instability in sporadic mucinous colorectal cancers. *Hum Pathol* 30:629-634, 1999

49. Hawkins NJ, Ward RL: Sporadic colorectal cancers with microsatellite instability and their possible origin in hyperplastic polyps and serrated adenomas. *J Natl Cancer Inst* 93:1307-1313, 2001

50. Tomlinson I, Bodmer W: Selection, the mutation rate and cancer: Ensuring that the tail does not wag the dog. *Nat Med* 5:11-12, 1999

51. Quinn E, Hawkins N, Yip L, et al: CD103+ intraepithelial lymphocytes: A unique population in microsatellite unstable sporadic colorectal cancer. *Eur J Cancer* 39:469-475, 2003

52. Whitehall VL, Wynter CV, Walsh MD, et al: Morphological and molecular heterogeneity within nonmicrosatellite instability-high colorectal cancer. *Cancer Res* 62:6011-6014, 2002

53. Smyrk TC, Watson P, Kaul K, et al: Tumor-infiltrating lymphocytes are a marker for microsatellite instability in colorectal carcinoma. *Cancer* 91:2417-2422, 2001