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ARTICLE

Tumor LINE-1 Methylation Level and Microsatellite Instability in Relation to Colorectal Cancer Prognosis

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Background Hypomethylation in long interspersed nucleotide element-1 (LINE-1) and high-degree microsatellite instability (MSI-high) in colorectal cancer (CRC) have been associated with inferior and superior survival, respectively; however, it remains uncertain whether the prognostic association of LINE-1 hypomethylation differs by MSI status. We hypothesized that the adverse prognostic association of LINE-1 hypomethylation might be stronger in MSI-high CRCs than in microsatellite stable (MSS) CRCs.

Methods Utilizing 1211 CRCs in the Nurses' Health Study and the Health Professionals Follow-up Study, we examined patient survival according to LINE-1 hypomethylation status in strata of MSI status. A Cox proportional hazards model was used to compute multivariable CRC-specific mortality hazard ratios (HRs) for a 10% decrease in LINE-1 methylation level (range = 23.1–93.1%), adjusting for potential confounders, including CpG island methylator phenotype, and *KRAS*, *BRAF*, and *PIK3CA* mutations. Statistical tests (log-rank test, chi-square test, and likelihood ratio test) were two-sided.

Results In MSI-high cancers, the association of LINE-1 hypomethylation with higher mortality (HR = 2.45, 95% confidence interval [CI] = 1.64 to 3.66, $P < .001$) was stronger than that in MSS cancers (HR = 1.10, 95% CI = 0.98 to 1.24, $P = .11$) ($P_{interaction} < .001$, between LINE-1 and MSI statuses). In MSI-high cases with CRC family history, the association of LINE-1 hypomethylation with higher mortality (HR = 5.13, 95% CI = 1.99 to 13.2; $P < .001$) was stronger than that in MSI-high cases without CRC family history (HR = 1.62, 95% CI = 0.89 to 2.94, $P = .11$) ($P_{interaction} = .02$, between LINE-1 and CRC family history statuses).

Conclusions The association of LINE-1 hypomethylation with inferior survival is stronger in MSI-high CRCs than in MSS CRCs. Tumor LINE-1 methylation level may be a useful prognostic biomarker to identify aggressive carcinomas among MSI-high CRCs.

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Colorectal cancer (CRC) represents a group of molecularly heterogeneous diseases characterized by differing sets of epigenetic and genetic abnormalities, including DNA hypomethylation in long interspersed nucleotide element-1 (LINE-1) and microsatellite instability (MSI) (1–4). LINE-1 constitutes a substantial portion (approximately 18%) of the human genome (5), and methylation level in LINE-1 correlates with global DNA methylation status (6). LINE-1 hypomethylated CRC has been associated with advanced stage or poor prognosis (7–12) and inversely with MSI-high independent of the CpG island methylator phenotype (CIMP) (13,14).

High degree of MSI due to deficiency of DNA mismatch repair is observed in approximately 15% of CRCs and is a well established prognostic biomarker for better survival (15–21). However, a subset of patients of MSI-high CRCs still succumbs to the disease, and hence, additional biomarkers are needed to further classify MSI-high CRCs into different prognostic groups. Recent evidence suggests that LINE-1 hypomethylation is associated with higher mortality in MSI-high CRCs (9); however, it remains uncertain whether the prognostic association of LINE-1 hypomethylation differs by MSI status. We hypothesized that the adverse prognostic association of LINE-1 hypomethylation might be stronger in MSI-high CRCs (characterized by numerous somatic mutations [22]) than in microsatellite stable (MSS) CRCs.

To test this hypothesis, we utilized a molecular pathological epidemiology database of 1211 CRCs in two prospective cohort studies

and examined patient survival according to tumor LINE-1 methylation level (scale 0–100%) in strata of MSI status. Considering the potential influence of Lynch syndrome (23,24), we additionally examined the prognostic role of tumor LINE-1 methylation level within MSI-high CRC cases in strata of CRC family history status.

Methods

Study Cohorts

We utilized two US nationwide, prospective cohort studies: the Nurses' Health Study (NHS, involving 121700 women who were enrolled in 1976) and the Health Professionals Follow-up Study (HPFS, involving 51529 men who were enrolled in 1986) (25). Every two years since these studies began, the participants have been sent follow-up questionnaires to update information on potential disease risk factors, and to identify newly diagnosed cancers and other diseases in themselves and their first-degree relatives. Lethal CRC cases were identified and confirmed by searching through the National Death Index. Study physicians reviewed all medical records related to CRC, extracted clinical information including stage and tumor location, and determined cause of death in deceased individuals. We collected paraffin-embedded tissue blocks from hospitals where participants with CRC had undergone tumor resection or diagnostic biopsy specimens.

Hematoxylin and eosin-stained tissue sections from all CRC cases were reviewed by a pathologist (S. Ogino) unaware of other data. Tumor differentiation was categorized as well to moderate vs poor (>50% vs ≤50% glandular area). We used available data on tumor LINE-1 methylation level, MSI status, and survival from 1211 CRC patients diagnosed up to 2008. Given the colorectal continuum model (26,27), we included both colon and rectal carcinomas in our primary analysis; we also examined colon cancer (excluding rectal cancer) in our secondary analysis. Patients were observed until death or January 1, 2012, whichever came first. The procedures and protocols of this study were approved by the institutional review boards for the Harvard School of Public Health and the Brigham and Women's Hospital. All subjects provided informed consent.

LINE-1 Methylation Analysis

DNA was extracted from archival tumor tissue. We performed bisulfite DNA treatment, polymerase chain reaction (PCR), and a pyrosequencing assay to quantify LINE-1 methylation level, after assay validation (28). We primarily used LINE-1 methylation level as a continuous variable (scale 0–100%) in survival analyses. To display some of our results, we categorized the degree of LINE-1 hypomethylation status into three groups, namely "severe" (<55% methylation), "intermediate" (55–64.9% methylation), and "mild/no" (≥65% methylation), consistent with our previous studies (29,30).

MSI Analysis

MSI analysis was performed utilizing a panel of 10 microsatellite markers, as previously described (20). MSI-high was defined as instability in greater than or equal to 30% of the markers, and MSS status as instability in less than 30% of the markers (20).

Analysis of CIMP, *KRAS*, *BRAF*, and *PIK3CA*

Assessment of CIMP, *KRAS*, *BRAF*, and *PIK3CA* of CRCs in our cohorts (25,31,32) is described in the Supplementary Methods (available online).

Statistical Analysis

All statistical analyses were carried out using SAS (version 9.3, SAS Institute, Cary, NC). All P values were two-sided. Statistical significance level was set at $P = .05$ for testing of our primary hypothesis of an interaction between LINE-1 methylation level (continuous) and MSI status (binary) in CRC-specific survival analysis. A statistical interaction was assessed by likelihood ratio test, which compared the model with the interaction term (of LINE-1 and MSI statuses) to the model without the interaction term. For secondary and exploratory analyses, we recognized multiple comparisons inherent in subgroup analyses and interpreted our data very cautiously to avoid overinterpretation. For demographic categorical data, the chi-square test was performed. A t-test or analysis of variance (ANOVA), assuming equal variances, was used to compare mean age.

Kaplan–Meier method and log-rank test were used for survival analyses. For analyses of CRC-specific mortality, deaths as a result of other causes were censored. To control for confounding, we used multivariable Cox proportional hazards regression models. In addition to the LINE-1 hypomethylation variable (continuous; 10% decrease as a unit), the multivariable model initially included sex, age at diagnosis (continuous), year of diagnosis (continuous), family history of CRC in first-degree relative(s) (present vs absent), tumor location (proximal colon vs distal colon vs rectum), tumor differentiation (well to moderate vs poor), CIMP (high vs low/negative), and *KRAS*, *BRAF*, and *PIK3CA* mutations. A single analysis model could estimate the effect of LINE-1 hypomethylation in each stratum of MSI status, using a reparameterization of the interaction term (of LINE-1 and MSI statuses), as previously described (33). To avoid overfitting, disease stage (I, II, III, IV, or unknown) (34) was used as a stratifying variable using the "strata" option in the SAS "proc phreg" command. A backward stepwise elimination was carried out with $P = .05$ as a threshold, to select variables for the final model. For cases with missing information in any of the categorical covariates (family history of CRC [0.4%], tumor location [1.1%], tumor differentiation [0.5%], CIMP [5.0%], *KRAS* [0.3%], *BRAF* [0.6%], and *PIK3CA* [7.3%]), we included these cases in the majority category of a given covariate. We confirmed that excluding cases with missing information in any of the covariates did not substantially alter results (data not shown). The proportionality of hazards assumption was assessed by a time-varying covariate (an interaction term of survival time and LINE-1 hypomethylation variable, $P > .15$).

Results

LINE-1 Hypomethylation and MSI Status in CRC

In 1211 incident CRCs within the two prospective cohort studies, the Nurses' Health Study and the Health Professionals Follow-up Study, we measured tumor LINE-1 methylation level (scale 0–100%), which ranged from 23.1% to 93.1% with mean of 62.7% and standard deviation of 9.4%. Normal colon mucosal tissue typically showed LINE-1 methylation level of 70% to 75% in our assay (14,28). There were 190 MSI-high CRCs and 1021 MSS CRCs. Table 1 shows characteristics of the 1211 cases of CRC, stratified by LINE-1 hypomethylation status and MSI status. Severe degree

Table 1. Clinical, pathologic, and molecular characteristics of 1211 colorectal cancer (CRC) cases stratified by tumor LINE-1 hypomethylation status and microsatellite instability (MSI) status

	MSS					MSI-high				
		LINE-1 hypomethylation status					LINE-1 hypomethylation status			
Clinical, pathologic, or molecular feature	**No. of cases**	**Mild/no (≥65% methylation)**	**Intermediate (55–64.9% methylation)**	**Severe (<55% (methylation)**	***P****	**No. of cases**	**Mild/no (≥65% methylation)**	**Intermediate (55–64.9% methylation)**	**Severe (<55% (methylation)**	***P****
No. of cases	1,021	376	423	222		190	125	48	17	
Sex					.14					.23
Male (HPFS)	495 (48%)	186 (49%)	191 (45%)	118 (53%)		65 (34%)	48 (38%)	13 (27%)	4 (24%)	
Female (NHS)	526 (52%)	190 (51%)	232 (55%)	104 (47%)		125 (66%)	77 (62%)	35 (73%)	13 (76%)	
Mean age ± SD, y	68.3 ± 8.9	68.9 ± 8.9	68.2 ± 8.7	67.3 ± 9.3	.11	70.4 ± 7.5	71.2 ± 7.5	70.3 ± 6.5	65.2 ± 8.5	.008
Year of diagnosis					<.001					<.001
Prior to 1995	342 (34%)	99 (26%)	155 (37%)	88 (40%)		43 (23%)	25 (20%)	13 (27%)	5 (29%)	
1995 to 1999	317 (31%)	105 (28%)	126 (30%)	86 (39%)		66 (35%)	31 (25%)	24 (50%)	11 (65%)	
2000 to 2008	362 (35%)	172 (46%)	142 (34%)	48 (22%)		81 (43%)	69 (55%)	11 (23%)	1 (5.9%)	
Family history of CRC in first-degree relative(s)					.15					.52
(−)	788 (78%)	303 (81%)	320 (76%)	165 (75%)		138 (73%)	94 (75%)	32 (67%)	12 (71%)	
(+)	228 (22%)	72 (19%)	100 (24%)	56 (25%)		52 (27%)	31 (25%)	16 (33%)	5 (29%)	
Tumor location					.09					.45
Cecum	161 (16%)	56 (15%)	69 (16%)	36 (17%)		44 (23%)	32 (26%)	9 (19%)	3 (18%)	
Ascending to transverse colon	235 (23%)	87 (24%)	103 (25%)	45 (21%)		121 (64%)	78 (62%)	33 (69%)	10 (59%)	
Splenic flexure to sigmoid colon	356 (35%)	114 (31%)	157 (37%)	85 (39%)		19 (10%)	13 (10%)	3 (6.3%)	3 (18%)	
Rectum	256 (25%)	113 (31%)	91 (22%)	52 (24%)		6 (3.2%)	2 (1.6%)	3 (6.3%)	1 (5.9%)	
Tumor differentiation					.04					.76
Well-moderate	958 (94%)	352 (94%)	404 (96%)	202 (91%)		131 (69%)	84 (67%)	35 (73%)	12 (71%)	
Poor	57 (5.6%)	22 (5.9%)	16 (3.8%)	19 (8.6%)		59 (31%)	41 (33%)	13 (27%)	5 (29%)	
Disease stage					.001					.20
I	246 (24%)	102 (27%)	106 (25%)	38 (17%)		39 (21%)	22 (18%)	12 (25%)	5 (29%)	
II	240 (24%)	81 (22%)	109 (26%)	50 (23%)		103 (54%)	73 (58%)	24 (50%)	6 (35%)	
III	290 (28%)	96 (26%)	123 (29%)	71 (32%)		30 (16%)	18 (14%)	10 (21%)	2 (12%)	
IV	148 (15%)	49 (13%)	52 (12%)	47 (21%)		10 (5.3%)	6 (4.8%)	1 (2.1%)	3 (18%)	
Unknown	97 (10%)	48 (13%)	33 (7.8%)	16 (7.2%)		8 (4.2%)	6 (4.8%)	1 (2.1%)	1 (5.9%)	
CIMP status					<.001					.10
CIMP-low/negative	904 (93%)	320 (89%)	376 (96%)	208 (97%)		45 (25%)	30 (25%)	8 (17%)	7 (44%)	
CIMP-high	64 (6.6%)	40 (11%)	17 (4.3%)	7 (3.3%)		137 (75%)	89 (75%)	39 (83%)	9 (56%)	
MLH1 promoter hypermethylation					.72					.01
(−)	949 (98%)	352 (98%)	387 (98%)	210 (98%)		40 (22%)	25 (21%)	7 (15%)	8 (50%)	
(+)	19 (2.0%)	8 (2.2%)	6 (1.5%)	5 (2.3%)		142 (78%)	94 (79%)	40 (85%)	8 (50%)	

(Table continues)

Table 1 (Continued).

Clinical, pathologic, or molecular feature	MSS					MSI-high				
		LINE-1 hypomethylation status					LINE-1 hypomethylation status			
	No. of cases	Mild/no (≥65% methylation)	Intermediate (55–64.9% methylation)	Severe (<55% (methylation)	*P**	No. of cases	Mild/no (≥65% methylation)	Intermediate (55–64.9% methylation)	Severe (<55% (methylation)	*P**
KRAS mutation					.80					.11
(–)	606 (59%)	225 (60%)	246 (58%)	135 (61%)		164 (87%)	104 (85%)	46 (96%)	14 (82%)	
(+)	413 (41%)	150 (40%)	176 (42%)	87 (39%)		24 (13%)	19 (15%)	2 (4.2%)	3 (18%)	
BRAF mutation					.08					.15
(–)	936 (92%)	337 (90%)	396 (94%)	203 (93%)		91 (48%)	57 (46%)	22 (46%)	12 (71%)	
(+)	79 (7.8%)	38 (10%)	25 (5.9%)	16 (7.3%)		98 (52%)	67 (54%)	26 (54%)	5 (29%)	
PIK3CA mutation					.36					.52
(–)	793 (84%)	292 (82%)	323 (83%)	178 (87%)		144 (83%)	96 (83%)	35 (80%)	13 (93%)	
(+)	156 (16%)	62 (18%)	67 (17%)	27 (13%)		30 (17%)	20 (17%)	9 (20%)	1 (7.1%)	

* The *P* value for statistical significance was adjusted for multiple hypothesis testing to $P = .05/24 = .002$. Thus, a *P* value between .05 and .002 should be regarded as of borderline statistical significance. CIMP = CpG island methylator phenotype; CRC = colorectal cancer; HPFS = Health Professionals Follow-up Study; LINE-1 = long interspersed nucleotide element-1; MSI = microsatellite instability; MSS = microsatellite stable; NHS = Nurses' Health Study; SD = standard deviation. (%) indicates the proportion of cases with a specific clinical, pathologic, or molecular feature among cancers with each LINE-1 methylation level in MSS- or MSI-high cases.

of LINE-1 hypomethylation was statistically significantly associated with higher disease stage ($P = .001$) and inversely associated with CIMP-high ($P < .001$) in MSS cases.

LINE-1 Hypomethylation, MSI Status, and CRC Mortality

Among 1211 patients, there were 648 deaths, including 356 CRC-specific deaths, during a median follow-up of 151 months (interquartile range: 110 to 204 months) for censored cases. We examined the relationship between LINE-1 hypomethylation and patient survival in all cases, and in strata of MSI status. In all CRC cases, tumor LINE-1 hypomethylation was associated with higher CRC-specific mortality in Kaplan–Meier analysis (log-rank $P < .001$) (Figure 1) and in univariate and multivariable Cox regression analyses (for 10% decrease in LINE-1 methylation: multivariable hazard ratio [HR] = 1.16, 95% confidence interval (CI) = 1.03 to 1.30, $P = .02$) (Table 2). For our main hypothesis testing, we examined statistical interaction between LINE-1 methylation level (continuous) and MSI status in CRC-specific survival analysis (Table 2), which revealed a statistically significant interaction ($P_{interaction} < .001$). In MSI-high CRCs, the association of LINE-1 hypomethylation with higher CRC-specific mortality was statistically significant (for 10% decrease in LINE-1 methylation: multivariable HR = 2.45, 95% CI = 1.64 to 3.66, $P < .001$) (Table 2). In MSS CRCs, the association of LINE-1 hypomethylation with CRC-specific mortality was weaker and not statistically significant (for 10% decrease in LINE-1 methylation: multivariable HR = 1.10, 95% CI = 0.98 to 1.24, $P = .11$) (Table 2). Figure 1 shows Kaplan–Meier survival curves according to LINE-1 hypomethylation categories in MSI-high CRCs and in MSS CRCs.

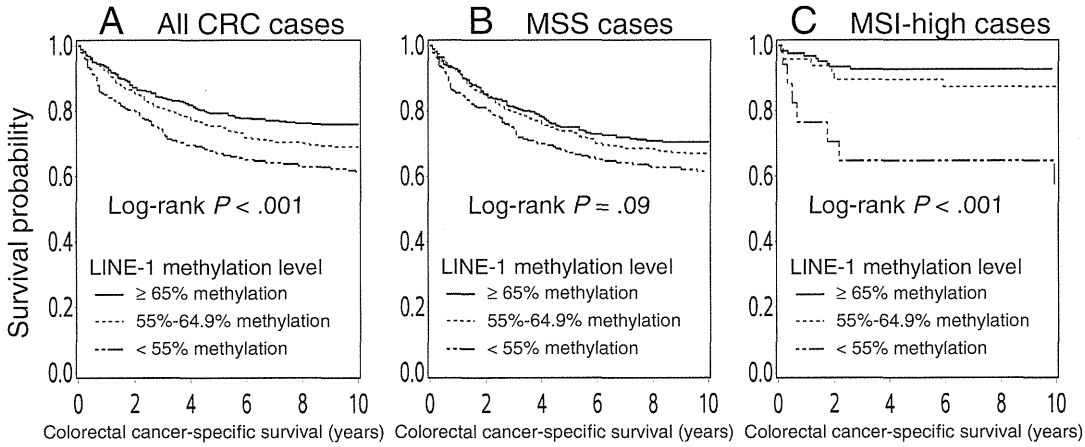
In secondary analyses of overall survival as an endpoint, there was a general trend toward differential prognostic associations of LINE-1 hypomethylation by MSI status, although the differences were not as evident as those in CRC-specific mortality analyses (Table 2; Supplementary Figure 1, available online). Results of Kaplan–Meier analyses according to combined LINE-1 hypomethylation and MSI status are provided in Supplementary Figure 2 (available online).

Table 3 shows secondary analyses limited to colon cancer. The association of LINE-1 hypomethylation with higher colon cancer-specific mortality was stronger in MSI-high colon cancer than in MSS colon cancer ($P_{interaction} < .001$, between LINE-1 and MSI statuses).

LINE-1 Hypomethylation, MSI/*BRAF* Status, and CRC Mortality

Although the utility of MSI/*BRAF* classification for prognostication in CRC has been demonstrated (20), it is an imperfect marker; ie, some patients with favorable MSI-high/*BRAF*–wild-type tumors may die of cancer, while other patients with unfavorable MSS/*BRAF*-mutant tumors may survive. Hence, additional markers are needed to refine prognostic groups of CRC.

As a secondary analysis, we examined the relationship between LINE-1 hypomethylation and patient survival in strata of MSI/*BRAF* subtype (Table 4). The association of LINE-1 hypomethylation with higher CRC-specific mortality appeared to be stronger in the MSI-high/*BRAF*–wild-type subtype (for 10% decrease in LINE-1 methylation; multivariable HR = 2.57, 95%



Number at risk

LINE-1 methylation level	Year					
	0	2	4	6	8	10
≥ 65%	501	432	390	320	265	212
55-64.9%	471	390	339	290	244	194
< 55%	239	188	161	142	128	110

Number at risk

LINE-1 methylation level	Year					
	0	2	4	6	8	10
≥ 65%	376	317	281	230	194	157
55-64.9%	423	347	298	252	214	171
< 55%	222	176	150	132	118	102

Number at risk

LINE-1 methylation level	Year					
	0	2	4	6	8	10
≥ 65%	125	115	109	90	71	55
55-64.9%	48	43	41	38	30	23
< 55%	17	12	11	10	10	8

Figure 1. Kaplan–Meier curves for colorectal cancer (CRC) patients according to tumor LINE-1 methylation level. CRC-specific survival in all CRC cases (**A**), microsatellite stable (MSS) CRC cases (**B**), and microsatellite instability (MSI)-high CRC cases (**C**). *P* value was calculated using log-rank test (two-sided). The tables (bottom) show the number of patients who remained alive and at risk of death at each time point after the diagnosis of CRC.

CI = 1.47 to 4.51, $P = .001$) than in the other three subtypes, namely MSS/*BRAF*–wild-type, MSS/*BRAF*-mutant, and MSI-high/*BRAF*-mutant (for 10% decrease in LINE-1 methylation; multivariable HR = 1.06, 95% CI = 0.93 to 1.21, $P = .36$; multivariable HR = 1.29, 95% CI = 0.94 to 1.77, $P = .12$; and multivariable HR = 1.28, 95% CI = 0.82 to 2.01, $P = .28$, respectively).

LINE-1 Hypomethylation, CRC Family History, and Mortality in MSI-High CRC

The MSI-high/*BRAF*–wild-type subgroup of CRCs encompasses Lynch syndrome cases, which are familial cancers due to a germline mutation in one of mismatch repair genes. As an exploratory analysis, we focused on MSI-high cases and examined the relationship between tumor LINE-1 hypomethylation and patient survival in strata of CRC family history status (Table 5). The association of LINE-1 hypomethylation with higher CRC-specific mortality appeared to be stronger in MSI-high cases with a family history of CRC in a first-degree relative (multivariable HR = 5.13, 95% CI = 1.99 to 13.2, $P < .001$) than in MSI-high cases without a family history of CRC in a first-degree relative (multivariable HR = 1.62, 95% CI = 0.89 to 2.94, $P = .11$) ($P_{interaction} = .02$, between LINE-1 methylation and CRC family history status in MSI-high cases) (Table 5). Nonetheless, these results must be interpreted cautiously, given the exploratory nature of this subgroup analysis and the low event numbers.

LINE-1 Hypomethylation, MSI Status, Tumor Location, and CRC Mortality

Considering the interactive prognostic association between MSI status and tumor location reported by Sinicrope et al. (35), we examined the relationship between LINE-1 hypomethylation and patient survival in strata of MSI status and tumor location (Supplementary Table 1, available online). The association of LINE-1 hypomethylation with mortality appeared to be modified by MSI status but not by tumor location, although statistical power was limited in this subgroup analysis.

Discussion

We conducted this study to test the hypothesis that the prognostic association of LINE-1 hypomethylation in CRC might be stronger in MSI-high CRCs than in MSS CRCs. Our data were consistent with this hypothesis, and the prognostic association of tumor LINE-1 hypomethylation indeed statistically significantly differed by MSI status. Although MSI-high is a well-established prognostic biomarker for better survival, a subset of MSI-high CRCs is lethal. In addition, our study has confirmed that tumor LINE-1 hypomethylation is associated with adverse prognosis in CRCs (7,8) and in MSI-high CRCs (9). Notably, our main hypothesis was that the prognostic association of tumor LINE-1 hypomethylation might be stronger in MSI-high CRCs (which are characterized by numerous somatic mutations [22]) than in MSS CRCs. This unique hypothesis has never been tested in the previous studies. To test our main hypothesis, it was necessary to utilize a large number of CRCs with detailed molecular analyses, including statuses of both MSI and LINE-1 hypomethylation, as well as other molecular features such as *KRAS*, *BRAF*, and *PIK3CA* mutations to control for possible confounding.

Examining tumor molecular and host factors has become increasingly important in CRC (36–42). LINE-1 methylation level and MSI are both important molecular markers in CRC. LINE-1 methylation level is used as a surrogate marker for global DNA methylation (43), and global DNA hypomethylation, indicated by LINE-1 hypomethylation, is associated with genomic

Table 2. Tumor LINE-1 hypomethylation and colorectal cancer (CRC) mortality, stratified by microsatellite instability (MSI) status

CRC subtype	No. of cases	CRC-specific mortality				Overall mortality			
		No. of events	Univariate HR (95% CI)	Stage-stratified HR (95% CI)	Multivariable HR* (95% CI)	No. of events	Univariate HR (95% CI)	Stage-stratified HR (95% CI)	Multivariable HR* (95% CI)
All CRC cases									
LINE-1 hypomethylation (10% decrease as a unit)	1211	356	1.24 (1.11 to 1.38)	1.19 (1.06 to 1.33)	1.16 (1.03 to 1.30)	648	1.01 (1.01 to 1.19)	1.07 (0.98 to 1.16)	1.06 (0.97 to 1.16)
P†			<.001	.003	.02		.04	.12	.18
MSS cases									
LINE-1 hypomethylation (10% decrease as a unit)	1021	333	1.13 (1.00 to 1.26)	1.10 (0.98 to 1.24)	1.10 (0.98 to 1.24)	562	1.04 (0.95 to 1.14)	1.03 (0.95 to 1.13)	1.02 (0.93 to 1.12)
P†			.05	.11	.11		.36	.47	.64
MSI-high cases									
LINE-1 hypomethylation (10% decrease as a unit)	190	23	1.90 (1.30 to 2.77)	2.23 (1.48 to 3.36)	2.45 (1.64 to 3.66)	86	1.33 (1.05 to 1.68)	1.38 (1.08 to 1.77)	1.42 (1.12 to 1.81)
P†			<.001	<.001	<.001		.02	.01	.004
$P_{interaction}$‡			.02	.002	<.001		.06	.03	.02

* The multivariable, stage-stratified Cox regression model initially included LINE-1 hypomethylation variable (continuous), sex, age at diagnosis, year of diagnosis, family history of CRC, tumor location, tumor differentiation, MSI status (only for all CRC cases), CpG island methylator phenotype, *KRAS*, *BRAF*, and *PIK3CA* mutations. CI = confidence interval; CRC = colorectal cancer; HR = hazard ratio; LINE-1 = long interspersed nucleotide element-1; MSI = microsatellite instability; MSS = microsatellite stable.

† *P* value was calculated using chi-square test (two-sided).

‡ $P_{interaction}$ value (between continuous LINE-1 methylation level and MSI status) was calculated using likelihood ratio test (two-sided), which compared the model with the interaction term to the model without the interaction term.

and chromosomal instability (14,44). LINE-1 hypomethylation in CRC may also be a marker for familial susceptibility to CRC (30,45). MSI is a well-established biomarker that is routinely used for assessment of familial CRC (Lynch syndrome) risk in combination with *BRAF* testing (23). LINE-1 hypomethylation is inversely associated with MSI-high, independent of CIMP (13,14), and LINE-1 hypomethylation (7–9), and MSI-high (15–21) are associated with inferior and superior prognosis, respectively.

To the best of our knowledge, this is among the first studies to address the interactive association between LINE-1 hypomethylation and MSI status in relation to the clinical outcome of CRC patients. Although some studies have shown the association of LINE-1 hypomethylation in CRCs with inferior survival, sample sizes of those studies would unlikely be adequate to analyze this interactive effect (sample size $N = 643$ [7] and $N = 161$ [8]). Rhee et al. (9) showed that LINE-1 hypomethylation was associated with adverse prognosis in MSI-high CRCs, but they did not examine MSS CRCs. A recent study has shown that LINE-1 hypomethylation is an independent prognostic biomarker in early-stage rectal cancer, but they did not examine colon cancer (46). Our resource of a large number of CRCs ($N = 1211$) has provided us with reasonable power to analyze this interactive association. Furthermore, we took into account other tumor molecular data, including CIMP, and *KRAS*, *BRAF*, and *PIK3CA* mutations.

Considering the utility of MSI/*BRAF* classification for prognostication in CRC (20), as well as its routine clinical use for familial risk assessment (23), we additionally examined the prognostic association of LINE-1 hypomethylation in strata of MSI/*BRAF* subtype. The association of LINE-1 hypomethylation with inferior survival appeared to be stronger in the MSI-high/*BRAF*–wild-type subtype, which is known to imply the most favorable subtype among the four MSI/*BRAF* subtypes (20). Although we should interpret the results cautiously, LINE-1 methylation level can potentially be used as an additional biomarker to refine the prognostic groups by MSI/*BRAF* classification.

Because the MSI-high/*BRAF*–wild-type CRC subgroup contains Lynch syndrome cases, we focused on MSI-high cases and examined the prognostic association of LINE-1 hypomethylation in strata of CRC family history status. The association of LINE-1 hypomethylation with inferior survival appeared to be stronger in MSI-high cases with a CRC family history than in MSI-high cases without a CRC family history. Nevertheless, we must interpret the results carefully to avoid overinterpretation, considering the exploratory nature of this analysis. Since the MSI-high/*BRAF*–wild-type subtype and the MSI-high subtype with a CRC family history are enriched with Lynch syndrome cases (23), these intriguing results warrant further investigation to examine whether LINE-1 hypomethylation serves as an unfavorable prognostic biomarker in Lynch syndrome cases.

It is interesting, but challenging, to speculate potential mechanisms of interaction between LINE-1 methylation level and MSI status. Compared to MSS tumor, MSI-high CRC characterized by numerous somatic mutations (22) might be more influenced by genomic DNA or LINE-1 hypomethylation, which is associated with chromosomal instability (14,44). Other possible mechanisms may involve inflammatory mediators (47–50), variation in locus-specific methylation patterns (43,51–54), and non-coding RNAs

Table 3. Tumor LINE-1 hypomethylation and colon cancer mortality, stratified by microsatellite instability (MSI) status

		Colon cancer-specific mortality				Overall mortality			
Colon cancer subtype	**No. of cases**	**No. of events**	**Univariate HR (95% CI)**	**Stage-stratified HR (95% CI)**	**Multivariable HR* (95% CI)**	**No. of events**	**Univariate HR (95% CI)**	**Stage-stratified HR (95% CI)**	**Multivariable HR* (95% CI)**
All colon cancer cases									
LINE-1 hypomethylation (10% decrease as a unit)	936	263	1.35 (1.20 to 1.53)	1.28 (1.12 to 1.45)	1.25 (1.09 to 1.44)	495	1.16 (1.06 to 1.28)	1.13 (1.03 to 1.24)	1.14 (1.03 to 1.26)
P†			<.001	<.001	.001		.002	.01	.01
MSS colon cancer cases									
LINE-1 hypomethylation (10% decrease as a unit)	752	241	1.21 (1.05 to 1.38)	1.16 (1.01 to 1.34)	1.18 (1.03 to 1.36)	413	1.10 (0.99 to 1.23)	1.03 (0.95 to 1.13)	1.02 (0.93 to 1.12)
P†			.007	.03	.02		.06	.47	.64
MSI-high colon cancer cases									
LINE-1 hypomethylation (10% decrease as a unit)	184	22	2.00 (1.36 to 2.93)	2.37 (1.55 to 3.62)	2.69 (1.77 to 4.11)	82	1.39 (1.09 to 1.78)	1.38 (1.08 to 1.77)	1.42 (1.12 to 1.81)
P†			<.001	<.001	<.001		.008	.01	.004
$P_{interaction}$‡			.02	.003	<.001		.10	.07	.05

* The multivariable, stage-stratified Cox regression model initially included LINE-1 hypomethylation variable (continuous), sex, age at diagnosis, year of diagnosis, family history of colorectal cancer, tumor differentiation, MSI status (only for all colon cancer cases), CpG island methylator phenotype, *KRAS*, *BRAF*, and *PIK3CA* mutations. CI = confidence interval; HR = hazard ratio; LINE-1 = long interspersed nucleotide element-1; MSI = microsatellite instability; MSS = microsatellite stable.

† *P* value was calculated using chi-square test (two-sided).

‡ $P_{interaction}$ value (between continuous LINE-1 methylation level and MSI status) was calculated using likelihood ratio test (two-sided), which compared the model with the interaction term with the model without the interaction term.

Table 4. Tumor LINE-1 hypomethylation and colorectal cancer (CRC) mortality, stratified by microsatellite instability (MSI) status and mutation status of *BRAF*

		CRC-specific mortality				Overall mortality			
CRC subtype	No. of cases	No. of events	Univariate HR (95% CI)	Stage-stratified HR (95% CI)	Multivariable HR* (95% CI)	No. of events	Univariate HR (95% CI)	Stage-stratified HR (95% CI)	Multivariable HR* (95% CI)
MSS/*BRAF*–wild-type									
LINE-1 hypomethylation (10% decrease as a unit)	936	291	1.12 (0.99 to 1.26)	1.08 (0.95 to 1.22)	1.06 (0.93 to 1.21)	506	1.02 (0.92 to 1.12)	1.00 (0.91 to 1.10)	1.00 (0.90 to 1.11)
P†			.08	.26	.36		.74	.99	.99
MSS/*BRAF*-mutant									
LINE-1 hypomethylation (10% decrease as a unit)	79	39	1.45 (1.05 to 2.01)	1.42 (1.04 to 1.94)	1.29 (0.94 to 1.77)	51	1.52 (1.13 to 2.05)	1.47 (1.10 to 1.96)	1.26 (0.95 to 1.69)
P†			.03	.03	.12		.006	.009	.11
MSI-high/*BRAF*–wild-type									
LINE-1 hypomethylation (10% decrease as a unit)	91	9	2.14 (1.26 to 3.64)	2.60 (1.44 to 4.70)	2.57 (1.47 to 4.51)	41	1.28 (0.92 to 1.79)	1.30 (0.90 to 1.88)	1.29 (0.90 to 1.84)
P†			.005	.002	.001		.14	.16	.16
MSI-high/*BRAF*-mutant									
LINE-1 hypomethylation (10% decrease as a unit)	98	14	1.03 (0.66 to 1.61)	1.25 (0.79 to 1.97)	1.28 (0.82 to 2.01)	45	1.10 (0.84 to 1.44)	1.23 (0.93 to 1.61)	1.30 (0.98 to 1.73)
P†			.90	.35	.28		.48	.14	.07

* The multivariable, stage-stratified Cox regression model initially included LINE-1 hypomethylation variable (continuous), sex, age at diagnosis, year of diagnosis, family history of CRC, tumor location, tumor differentiation, CpG island methylator phenotype, *KRAS* and *PIK3CA* mutations. CI = confidence interval; CRC = colorectal cancer; HR = hazard ratio; LINE-1 = long interspersed nucleotide element-1; MSI = microsatellite instability; MSS = microsatellite stable.

† *P* value was calculated using chi-square test (two-sided).

Table 5. Microsatellite instability (MSI)-high colorectal cancer (CRC) mortality according to tumor LINE-1 hypomethylation status, stratified by family history of CRC in first-degree relative(s)

		CRC-specific mortality				Overall mortality			
MSI-high CRC subtype	No. of cases	No. of events	Univariate HR (95% CI)	Stage-stratified HR (95% CI)	Multivariable HR* (95% CI)	No. of events	Univariate HR (95% CI)	Stage-stratified HR (95% CI)	Multivariable HR* (95% CI)
Family history of CRC (–)									
LINE-1 hypomethylation (10% decrease as a unit)	138	16	1.36 (0.82 to 2.25)	1.41 (0.81 to 2.47)	1.62 (0.89 to 2.94)	61	1.17 (0.88 to 1.55)	1.18 (0.88 to 1.59)	1.21 (0.90 to 1.63)
P†			.24	.23	.11		.29	.27	.21
Family history of CRC (+)									
LINE-1 hypomethylation (10% decrease as a unit)	52	7	6.39 (2.45 to 16.7)	4.89 (1.93 to 12.4)	5.13 (1.99 to 13.2)	25	1.95 (1.12 to 3.40)	1.89 (1.10 to 3.25)	2.21 (1.27 to 3.86)
P†			<.001	<.001	<.001		.02	.02	.005
$P_{interaction}$‡			.003	.02	.02		.10	.13	.05

* The multivariable, stage-stratified Cox regression model initially included LINE-1 hypomethylation variable (continuous), sex, age at diagnosis, year of diagnosis, tumor location, tumor differentiation, CpG island methylator phenotype, *KRAS*, *BRAF*, and *PIK3CA* mutations. CI = confidence interval; CRC = colorectal cancer; HR = hazard ratio; LINE-1 = long interspersed nucleotide element-1; MSI = microsatellite instability.

† *P* value was calculated using chi-square test (two-sided).

‡ $P_{interaction}$ value (between continuous LINE-1 methylation level and binary status of CRC family history) was calculated using likelihood ratio test (two-sided), which compared the model with the interaction term with the model without the interaction term.

(55–57). Further studies are required to elucidate the underlying mechanisms of the interactive prognostic association between tumor LINE-1 methylation level and MSI status.

Some limitations of our study deserve discussion. Because data on cancer treatment were limited, unknown bias, including differential treatment assignment, might confound results. In survival analyses, we adjusted for disease stage, on which treatment decisions are mainly based. Another caveat relates to the study population. Health professionals may not be completely representative of the general US population. Nonetheless, the pathologic and molecular features of our CRC cases are generally compatible with data from the US cancer registry and published literature. Another limitation is that we excluded cases without available tumor data, which might cause bias. Nonetheless, a previous study has shown that there are no statistically significant demographic or clinical differences between cases with and without available tumor data (58). Finally, we need to replicate the findings before implementing tumor LINE-1 methylation measurement as a clinical test following the guidelines (59).

Strengths of this study include the use of data from the two US nationwide, prospective cohort studies. Information on cancer staging, family history of CRC, and other clinicopathologic and tumor molecular data was integrated into the molecular pathological epidemiology (60,61) database. Our cohort participants were treated at hospitals throughout the US and were more representative of CRC cases in the general US population than patients in only a few academic hospitals. Finally, by virtue of our database, we could assess the prognostic association of LINE-1 hypomethylation in strata of MSI status, while controlling for multiple potential confounders, including disease stage, CIMP, and *KRAS*, *BRAF*, and *PIK3CA* mutations.

In conclusion, we showed a stronger association of LINE-1 hypomethylation with inferior survival in MSI-high CRCs than in MSS CRCs, further attesting biological heterogeneity of MSI-high CRCs. LINE-1 methylation may be a useful prognostic biomarker to identify aggressive cancer cases among generally indolent MSI-high CRCs.

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ORIGINAL ARTICLE – COLORECTAL CANCER

SMO Expression in Colorectal Cancer: Associations with Clinical, Pathological, and Molecular Features

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ABSTRACT

Background. Smoothened, frizzled family receptor (SMO) is an important component of the hedgehog signaling pathway, which has been implicated in various human carcinomas. However, clinical, molecular, and prognostic associations of SMO expression in colorectal cancer remain unclear.

Methods. Using a database of 735 colon and rectal cancers in the Nurse's Health Study and the Health Professionals Follow-up Study, we examined the relationship of tumor SMO expression (assessed by immunohistochemistry) to prognosis, and to clinical, pathological, and tumor molecular features, including mutations of *KRAS, BRAF,* and *PIK3CA*, microsatellite instability, CpG island methylator phenotype (CIMP), LINE-1 methylation, and expression of phosphorylated AKT and CTNNB1.

Results. SMO expression was detected in 370 tumors (50 %). In multivariate logistic regression analysis, SMO expression was independently inversely associated with phosphorylated AKT expression [odds ratio (OR) 0.48; 95 % confidence interval (CI) 0.34–0.67] and CTNNB1 nuclear localization (OR 0.48; 95 % CI 0.35–0.67). SMO expression was not significantly associated with colorectal cancer-specific or overall survival. However, in CIMP-high tumors, but not CIMP-low/0 tumors, SMO expression was significantly associated with better colorectal cancer-specific survival (log-rank $P = 0.012$; multivariate hazard ratio, 0.36; 95 % CI 0.13–0.95; $P_{\text{interaction}} = 0.035$, for SMO and CIMP status).

Conclusions. Our data reveal novel potential associations between the hedgehog, the WNT/CTNNB1, and the PI3K (phosphatidylinositol-4,5-bisphosphonate 3-kinase)/AKT pathways, supporting pivotal roles of SMO and hedgehog signaling in pathway networking. SMO expression in colorectal cancer may interact with tumor CIMP status to affect patient prognosis, although confirmation by future studies is needed.

Colorectal cancers represent a heterogeneous group of complex multifactorial diseases, which are influenced by

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host and environmental factors.[1] Molecular classification [e.g., by *KRAS, BRAF,* and microsatellite instability (MSI) status] has become essential in both research and clinical practice to better predict tumor progression and behavior.[2–5]

The hedgehog signaling pathway plays a role in patterning, growth, and differentiation in various tissues, including the gastrointestinal tract.[6–8] In mammals, hedgehog signaling is initiated through binding of one of three ligands [sonic hedgehog (SHH), Indian hedgehog (IHH), and desert hedgehog (DHH)] to the transmembrane receptor patched 1 (PTCH1), leading to release of the suppressed transmembrane protein smoothened, fizzled family receptor (SMO) and subsequent activation of GLI transcription factors.[6] Hedgehog signaling has been implicated in the pathogenesis of various human cancers, either through hedgehog ligand-dependent activation, or through ligand-independent activation, i.e., by loss-of-function mutations in *PTCH1* or gain-of-function mutations in the proto-oncogene *SMO.*[9–11] Consequently, the hedgehog pathway is viewed as a potential therapeutic target.[9,12]

Although evidence supporting a role of the hedgehog pathway in colorectal neoplasia has tended to be inconsistent, accumulating experimental data demonstrate that the hedgehog signaling pathway cooperates with other molecular alternations and signaling pathways, such as WNT signaling, and phosphatidylinositol-4,5-biphosphonate 3-kinase (PI3K)/AKT, in multiple tumorigenic contexts, even in the absence of hedgehog ligand-dependent pathway activation.[13–26]

Given evidence of cross-talk between hedgehog and other signaling pathways in human carcinogenesis, we hypothesized that SMO expression in colorectal cancer might be associated with other important tumor characteristics, such as CTNNB1 and phosphorylated AKT expression. We therefore used a molecular pathological epidemiology database,[27,28] derived from colorectal cancers arising in two U.S. nationwide prospective cohort studies, to examine SMO expression status in colorectal cancer, and to assess the relationships between SMO expression and other important molecular features, including MSI; CpG island methylation phenotype (CIMP); long interspersed nucleotide element-1 (LINE-1) methylation; and *KRAS, BRAF,* and *PIK3CA* mutations. We also sought to evaluate the prognostic association of SMO expression, and to explore the potential for its interaction with other tumor features in survival analyses.

MATERIALS AND METHODS

Study Group

We used the database of two prospective cohort studies, the Nurses' Health Study (NHS, $N = 121{,}700$ women observed since 1976) and the Health Professionals Follow-Up Study (HPFS, $N = 51{,}500$ men observed since 1986).[29] Participants were sent follow-up biennial questionnaires to update information on diet and lifestyle factors, and to identify newly diagnosed cancers and other diseases. In this population-based study, besides medications that a given patient took by themselves, treatment modality was chosen by treating physicians, and detailed treatment data were not available. After confirmation of colorectal cancer, we requested paraffin embedded tissue blocks from hospitals across the United States, where participants had undergone resection of primary tumors. We were able to obtain colorectal cancer specimens for 1,443 cases out of 3,019 colorectal cancer cases recorded up to June 2006. Diagnostic biopsy specimens from rectal cancer patients who received preoperative therapy were collected in order to avoid treatment-related artifact. Tumor location was categorized [cecum; ascending colon (including hepatic flexure); transverse colon; descending colon (including splenic flexure); sigmoid colon; rectum] based on medical records.[30] All colorectal cancer cases were confirmed through review of histology by a pathologist (S.O.) blinded to exposure data. Tumor grade was categorized as high (≤50 % glandular area) or low (>50 % glandular area). On the basis of the availability of SMO expression data and survival data, a total of 735 colorectal cancer cases diagnosed up to 2006 were included in this study. Patients were observed until death, or January 2011, whichever came first. Death of a participant was ascertained through the National Death Index, or by reporting by family members or postal authorities. The cause of death was assigned by study physicians. Written informed consent was obtained from all study subjects. Human subjects committees at Harvard School of Public Health and Brigham and Women's Hospital approved this study.

Immunohistochemistry for SMO, Phosphorylated AKT, and CTNNB1

Tissue microarray blocks were constructed as previously described.[31] Methods of immunohistochemical staining and interpretations for phosphorylated AKT (at amino acid position Ser 473) and CTNNB1 have been described previously.[32–34] For SMO immunostaining, deparaffinized tissue sections were heated in a microwave for 15 min in Antigen Retrieval Citra Solution, pH 6 (BioGenex Laboratories, San Ramon, CA). Tissue sections were incubated with dual endogenous enzyme block (Dako, Carpinteria, CA), then serum free protein block (Dako), each for 15 min. Slides were incubated at room temperature for 1 h with a primary antibody against SMO (1:100, rabbit polyclonal, sc-13943; Santa Cruz, San Diego, CA). Envision anti-rabbit HRP-labeled polymer (Dako) was applied to the sections for 30 min, followed by visualization using

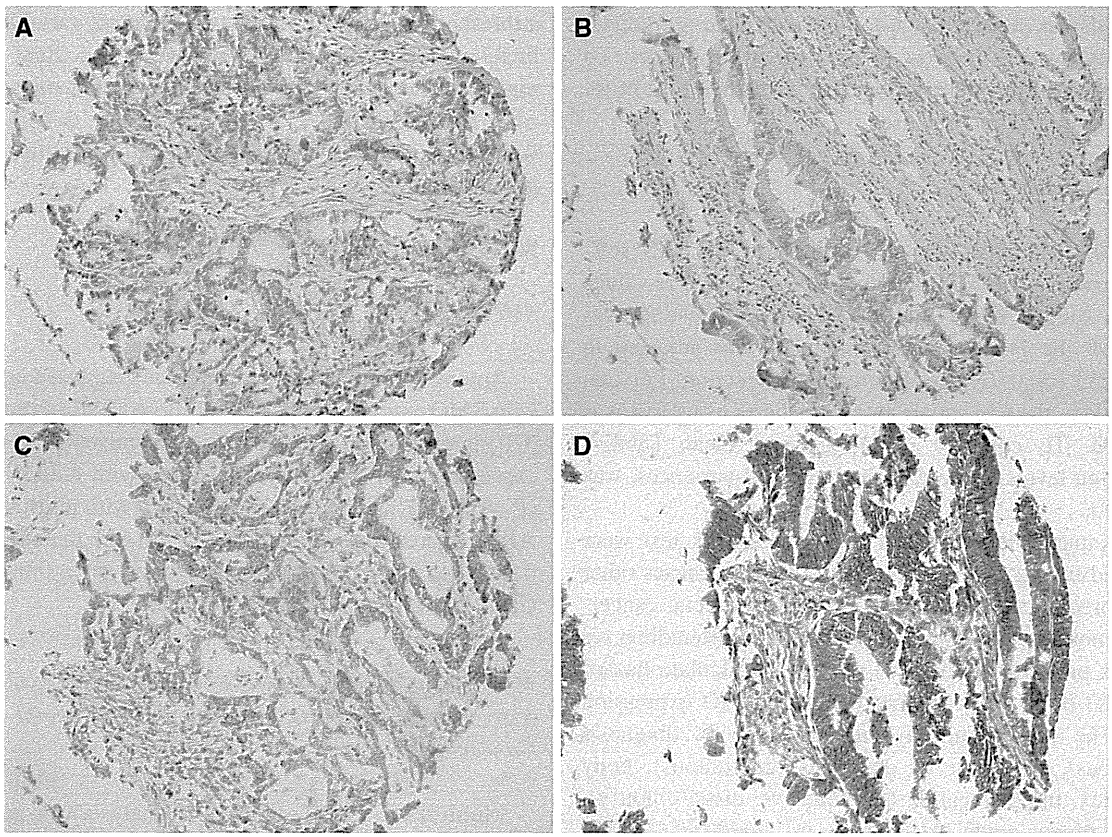

FIG. 1 SMO expression in colorectal cancer. No expression (**a**), weak expression (**b**), moderate expression (**c**), and intense expression (**d**) in colorectal cancer cells

the chromogen 3,3-diaminobenzidine (Dako) and hematoxylin counterstain. The specificity of the SMO antibody was confirmed by previous studies in different tissues and cells.[35–37] Positive and negative controls were included in each panel of immunohistochemistry for all markers.[33,34] Known positive prostate carcinoma was used as a positive control for SMO.[38] Sections processed with replacement of primary antibody by Tris-buffered saline were used as a negative control.

For each case, cytoplasmic SMO status was recorded as absent, weak, moderate, or intense staining. SMO expression was defined as the presence of weak to intense staining (Fig. 1). Immunostained tissue for each marker was scored by a single pathologist (SMO by X.L.; phosphorylated AKT by Y.B.; and CTNNB1 by T.M.) blinded to other data. A subset sample of over 100 cases for each marker was scored independently by a second pathologist (SMO by T.M.; phosphorylated AKT by K.S.; and CTNNB1 by S.O.) unaware of other data. The concordance between the two observers (all $P < 0.0001$) was 0.91 $\kappa = 0.79, n = 118$) for SMO, 0.81 ($\kappa = 0.59$, $n = 132$) for phosphorylated AKT, and 0.90 ($\kappa = 0.80$, $n = 292$) for nuclear CTNNB1 localization, indicating good to substantial agreement.

Sequencing of KRAS, BRAF, and PIK3CA Mutation, and Analysis for MSI

Genomic DNA was extracted from paraffin-embedded tissue. PCR and pyrosequencing targeted at *KRAS* [codons 12 and 13 (because 90 % of *KRAS* mutations occur in these two codons)], *BRAF* (codon 600), and *PIK3CA* exons 9 and 20 were performed as previously described.[32,39–42] MSI was assessed using a panel of 10 microsatellite markers (D2S123, D5S346, D17S250, BAT25, BAT26, BAT40, D18S55, D18S56, D18S67, and D18S487).[41] MSI-high was defined as the presence of instability in 30 % or more of the markers, and MSI-low/microsatellite stability (MSS) as instability 0–29 % of the markers.[41]

Real-Time PCR for CpG Island Methylation and Pyrosequencing to Measure LINE-1 Methylation

Sodium bisulfite treatment of DNA, and real-time PCR assays (MethyLight) were performed as previously described.[41,43,44] We quantified promoter methylation at eight CIMP-specific loci: *CACNA1G, CDKN2A* (p16), *CRABP1, IGF2, MLH1, NEUROG1, RUNX3,* and *SOCS1.*

CIMP-high was defined as ≥6 (of 8) methylated promoters, and CIMP-low/0 as 0–5 (of 8) methylated promoters. To accurately quantify methylation level in LINE-1, a PCR-pyrosequencing assay was used.[45,46]

Statistical Analysis

All statistical analyses were performed by SAS software, version 9.2 (SAS Institute, Cary, NC). All P values were two-sided. When multiple hypothesis testing was performed, the P value for significance was adjusted to $P = 0.0033$ (=0.05/15) by Bonferroni correction. For categorical data, the Chi square test or Fisher's exact test was performed. To compare mean age and mean LINE-1 methylation levels, a t test, assuming equal variances, was performed.

The Kaplan–Meier method and the log-rank test were performed for survival analyses. Deaths from causes other than colorectal cancer were censored in colorectal cancer-specific mortality analyses. To control for confounding, we used Cox proportional hazards models to calculate hazard ratio (HR) of death according to tumor SMO expression status. The model initially included age at diagnosis (continuous), sex, year of diagnosis (continuous), body mass index, tumor location (proximal vs. distal colon vs. rectum), tumor grade, MSI (high vs. low/MSS), CIMP (high vs. low/0), LINE-1 methylation (continuous), *BRAF* mutation, *KRAS* mutation, and *PIK3CA* mutation, in addition to CTNNB1 and phosphorylated AKT expression. To minimize residual confounding and overfitting, disease stage (I, II, III, IV, or unknown) was used as a stratifying variable using the "strata" option in the SAS "proc phreg" command. To avoid overfitting, variables in the final model were selected using backward stepwise elimination with a threshold of $P = 0.05$. Interaction was assessed using the Wald test on the cross-product of SMO and another variable of interest (excluding cases missing data) in a multivariate Cox model. To improve efficiency of the models, cases with missing data in any of the categorical variables [CIMP (1.8 %), MSI (2.0 %), *BRAF* (1.3 %), *KRAS* (1.1 %), *PIK3CA* (9.8 %), CTNNB1 (4.7 %), and phosphorylated AKT (6.9 %)], were included in the majority category for that variable. We confirmed that excluding cases with missing information in any of the covariates did not substantially alter the results (data not shown).

To assess whether associations between SMO expression and the variablesQuery in Table 1 were independent of other variables, a multivariate logistic regression analysis was conducted for cross-sectional analyses. To calculate adjusted odds ratios (OR), the model initially included variables as in Cox proportional hazards models. To avoid overfitting, a backward stepwise elimination with a threshold of $P = 0.05$ was used to select variables in the final model. After the variables in the final logistic regression model were selected, we used a missing indicator method for those cases with missing data in a given variable to obtain a more accurate effect estimate in the given variable.

RESULTS

SMO Expression in Colorectal Cancer

Among 735 colorectal cancer cases diagnosed up to 2006 with SMO expression data, we observed SMO expression in 370 tumors (50 %) by immunohistochemistry (Fig. 1). SMO expression was positively associated with *KRAS* mutation ($P = 0.0027$) and inversely associated with phosphorylated AKT expression ($P < 0.0001$), *BRAF* mutation ($P = 0.0026$), CTNNB1 nuclear localization ($P = 0.0005$), and CIMP-high status ($P = 0.0035$) (Table 1).

Multivariate Logistic Regression Analysis to Assess Associations with SMO Expression in Colorectal Cancer

Multivariate logistic regression analysis was performed to assess independent relationships between SMO expression and other factors. Phosphorylated AKT expression [multivariate OR 0.48; 95 % confidence interval (CI) 0.34–0.67; $P < 0.0001$] and CTNNB1 nuclear localization (OR 0.48; 95 % CI 0.35–0.67; $P < 0.0001$) remained significantly associated with SMO expression in the final model.

In addition, *BRAF* mutation/*KRAS* wild type (vs. *BRAF* wild type/*KRAS* wild type) and CIMP-high remained in the final model [(OR 0.49; 95 % CI 0.28–0.85; $P = 0.011$) and (OR 0.59; 95 % CI 0.35–0.98; $P = 0.043$), respectively], but these associations were not statistically significant given multiple hypothesis testing (Table 2).

SMO Expression and Patient Survival in Colorectal Cancer

During follow-up of 735 patients with survival data (median follow-up time 14.1 years for censored cases), there were 373 deaths, including 216 deaths due to colorectal cancer. In Kaplan–Meier analyses, SMO expression was not significantly associated with colorectal cancer-specific survival (log-rank $P = 0.85$) or overall survival (log-rank $P = 0.72$).

We performed Cox proportional hazards regression models to assess mortality according to SMO status, but we did not observe a significant association between SMO

TABLE 1 Clinical, pathological, and molecular features of colorectal cancer according to SMO expression status

Feature	Total	SMO nonexpression	SMO expression	*P*
Total no.	735	365	370	
Sex				0.093
Male (HPFS)	271 (37 %)	146 (40 %)	125 (34 %)	
Female (NHS)	464 (63 %)	219 (60 %)	245 (66 %)	
Age at diagnosis, *y*, mean ± SD	67.2 ± 8.4	67.4 ± 8.1	67.0 ± 8.7	0.60
Family history of colorectal cancer in first-degree relatives				0.20
Absent	587 (80 %)	299 (82 %)	288 (78 %)	
Present	148 (20 %)	66 (18 %)	82 (22 %)	
Body mass index				0.31
<30 kg/m^2	591 (81 %)	299 (82 %)	292 (79 %)	
≥30 kg/m^2	142 (19 %)	65 (18 %)	77 (21 %)	
Tumor location				0.60
Cecum	129 (18 %)	64 (18 %)	65 (17 %)	
Ascending colon	156 (21 %)	78 (22 %)	78 (21 %)	
Transverse colon	75 (10 %)	41 (11 %)	34 (9 %)	
Descending colon	59 (8 %)	34 (9 %)	25 (7 %)	
Sigmoid colon	165 (23 %)	77 (21 %)	88 (24 %)	
Rectum	148 (20 %)	68 (19 %)	80 (22 %)	
Disease stage				0.46
I	158 (21 %)	82 (22 %)	76 (20 %)	
II	227 (31 %)	108 (30 %)	119 (32 %)	
III	207 (28 %)	96 (26 %)	111 (30 %)	
IV	102 (14 %)	55 (15 %)	47 (13 %)	
Unknown	41 (6 %)	24 (7 %)	17 (5 %)	
Tumor grade				0.044
Low	664 (90 %)	321 (88 %)	343 (93 %)	
High	70 (10 %)	43 (12 %)	27 (7 %)	
MSI status				0.087
Low/MSS	602 (84 %)	288 (81 %)	314 (86 %)	
High	118 (16 %)	67 (19 %)	51 (14 %)	
CIMP status				0.0035
Low/0	604 (84 %)	284 (80 %)	320 (88 %)	
High	118 (16 %)	73 (20 %)	45 (12 %)	
BRAF status				0.0026
Wild type	616 (85 %)	293 (81 %)	323 (89 %)	
Mutant	109 (15 %)	69 (19 %)	40 (11 %)	
KRAS status				0.0027
Wild type	461 (63 %)	248 (69 %)	213 (58 %)	
Mutant	266 (37 %)	112 (31 %)	154 (42 %)	
PIK3CA status				0.92
Wild type	556 (84 %)	275 (84 %)	281 (84 %)	
Mutant	107 (16 %)	54 (16 %)	53 (16 %)	
LINE-1 methylation level, %, mean ± SD	61.3 ± 9.4	61.7 ± 10.0	61.0 ± 8.8	0.33
CTNNB1 nuclear localization				0.0005
Negative	372 (53 %)	159 (46 %)	213 (60 %)	
Positive	329 (47 %)	184 (54 %)	145 (40 %)	
Phosphorylated AKT expression				<0.0001
Negative	253 (37 %)	99 (29 %)	154 (45 %)	

TABLE 1 continued

Feature	Total	SMO nonexpression	SMO expression	*P*
Positive	431 (63 %)	240 (71 %)	191 (55 %)	

Percentages indicate proportion of patients with specific clinical, pathological, or molecular feature among all patients, or patients with specific tumor SMO expression status

SMO smoothened, frizzled family receptor, *CIMP* CpG island methylator phenotype, *HPFS* Health Professionals Follow-up Study, *LINE-1* long interspersed nucleotide element 1, *MSI* microsatellite instability, *MSS* microsatellite stable, *NHS* Nurses' Health Study, *SD* standard deviation

TABLE 2 Multivariate logistic regression analysis to calculate adjusted OR for association of variable with SMO expression

Variable in the final multivariate model	Multivariate OR (95 % CI)	*P*
Phosphorylated AKT expression	0.48 (0.34–0.67)	<0.0001
CTNNB1 nuclear localization	0.48 (0.35–0.67)	<0.0001
BRAF/*KRAS* status		
BRAF mutation/*KRAS* wild type (vs. *BRAF* wild type/*KRAS* wild type)	0.49 (0.28–0.85)	0.011
BRAF wild type/*KRAS* mutation (vs. *BRAF* wild type/*KRAS* wild type)	1.35 (0.96–1.89)	0.082
CIMP-high (vs. low/0)	0.59 (0.35–0.98)	0.043

Multivariate logistic regression model initially included age, sex, year of diagnosis, body mass index, tumor location, family history, MSI, CpG island methylator phenotype, *KRAS*, *BRAF*, and *PIK3CA* mutation, LINE-1 methylation, CTNNB1 nuclear localization, and phosphorylated AKT expression. Backward elimination with threshold of $P = 0.05$ was used to select variables in the final model. When multiple hypothesis testing was performed, *P* value for significance was adjusted to 0.0033 (=0.05/15) by Bonferroni correction

OR odds ratio, *SMO* smoothened, frizzled family receptor, *CI* confidence interval

expression and survival in univariate, stage-stratified, or multivariate stage-stratified analyses (data not shown).

Interactions Between SMO Expression and Other Variables in Colorectal Cancer Survival Analysis

We examined whether any clinical, pathological, or molecular variables significantly modified the association of SMO expression with patient survival. We observed a borderline significant interaction between SMO expression and CIMP status in colorectal cancer-specific survival ($P_{interaction} = 0.035$, given multiple testing significance level was adjusted to $P = 0.0033$). For patients with CIMP-high tumor, SMO positivity was significantly associated with better colorectal cancer-specific survival (multivariate HR 0.36, 95 % CI 0.13–0.95), whereas for patients with CIMP-low/0 tumor, SMO positivity was not significantly associated with colorectal cancer-specific survival (Table 3). The differential effect of SMO expression on colorectal cancer-specific survival according to CIMP status was also evident in Kaplan–Meier analyses (Fig. 2).

The association of SMO expression with cancer-specific mortality did not significantly differ according to any of the other variables.

DISCUSSION

In this study, the unique resource of a molecular pathological epidemiology database, containing a large number of colorectal cancers and prospectively collected data from two cohort studies, enabled us to comprehensively evaluate the associations of SMO expression with clinical, pathological, and tumor molecular features.[27,28] We observed that SMO was expressed in approximately half of colorectal cancers. In a multivariate logistic regression model, SMO expression was significantly inversely associated with phosphorylated AKT expression and CTNNB1 nuclear localization. An inverse association was also observed between SMO expression and *BRAF* mutant/*KRAS* wild type; however, the association was of borderline significance when multiple testing was taken into account.

Recent studies have demonstrated that colorectal cancers constitute a group of heterogeneous tumors at the molecular level.[47,48] The development and progression of colorectal neoplasia is attributable to the accumulation of genetic and epigenetic changes and the complex interaction of aberrations in various signaling pathways.[49–52] Each tumor has its own unique characteristics in terms of molecular phenotype, tumor microenvironment, and interactomes within and between neoplastic and host cells.[1,53] Therefore, tumor biomarker testing contributes to personalized medicine research and ultimately to clinical practice.[50,54–56]

Experimental data suggest a link between SMO expression and the PI3K/AKT pathway. AKT is a major downstream effector of PI3K and plays a crucial role in regulating a wide variety of cellular process, including cellular metabolism as well as cell proliferation and survival.[57] Riobo et al.[24] have previously shown that PI3K and AKT are essential for SHH signaling. Furthermore, SMO activity is required for cooperation between SHH and insulinlike growth factor in promoting myogenic proliferation and differentiation via the MAPK/ERK and PI3K/AKT

TABLE 3 SMO expression status in colorectal cancer and patient mortality in strata of CpG island methylator phenotype status

Tumor characteristic	*n*	Colorectal cancer-specific mortality				Overall mortality			
		No. of events	Univariate HR (95 % CI)	Stage-stratified HR (95 % CI)	Multivariate stage-stratified HR (95 % CI)	No. of events	Univariate HR (95 % CI)	Stage-stratified HR (95 % CI)	Multivariate stage-stratified HR (95 % CI)
CIMP-low/0									
No expression	274	75	1 (referent)	1 (referent)	1 (referent)	131	1 (referent)	1 (referent)	1 (referent)
Expression	304	95	1.15 (0.85–1.56)	1.11 (0.82–1.51)	1.08 (0.79–1.47)	156	1.10 (0.87–1.39)	1.09 (0.86–1.38)	1.02 (0.80–1.29)
CIMP-high									
No expression	73	23	1 (referent)	1 (referent)	1 (referent)	39	1 (referent)	1 (referent)	1 (referent)
Expression	45	5	0.30 (0.11–0.78)	0.37 (0.14–0.98)	0.36 (0.13–0.95)	23	0.77 (0.46–1.30)	0.87 (0.51–1.47)	0.73 (0.43–1.23)
P for interaction (SMO expression and CIMP status)			0.0090	0.035	0.035		0.22	0.44	0.26

Multivariate, stage-stratified Cox regression model initially included age, sex, year of diagnosis, body mass index, tumor location, tumor grade, MSI, *KRAS, BRAF,* and *PIK3CA* mutation, LINE-1 methylation, CTNNB1 nuclear localization, and phosphorylated AKT expression. Backward elimination with threshold of $P = 0.05$ was used to select variables in final models

SMO smoothened, frizzled family receptor, *HR* hazard ratio, *CI* confidence interval

pathways.[25] In our data set, SMO expression was inversely associated with phosphorylated AKT expression in colorectal cancer, suggesting that SMO activation may tend to be mutually exclusive with AKT activation in colorectal cancer development.

Cross-talk between the hedgehog and WNT signaling pathways in intestinal tumorigenesis remains controversial.[7,20,22,50,58] Several groups have reported possible negative regulation of the WNT pathway by hedgehog signaling.[7,58] In one study, overexpression of IHH resulted in down-regulation of intestinal CTNNB1.[58] Nuclear expression of CTNNB1 has been found to be inversely associated with GLI1 staining in colorectal cancers, suggesting that GLI1 plays an inhibitory role in the development of colorectal cancer driven by WNT signaling.[20] However, Arimura et al.[22] have shown that reduced SMO expression inhibits WNT signaling by down-regulating nuclear CTNNB1 expression independent of GLI-mediated hedgehog signaling. Our current findings suggest that tumors with SMO expression are inversely associated with CTNNB1 nuclear expression, favoring a negative regulation of the WNT pathway by hedgehog signaling.

Although SMO expression was not associated with colorectal cancer-specific survival or overall survival, our data suggest a possible interaction with CIMP status in patient prognosis. CIMP constitutes an epigenomic phenomenon characterized by widespread promoter methylation, which leads to tumor suppressor gene silencing.[59] CIMP status has been extensively investigated in colorectal cancer.[60–65] In our current study, we observed that SMO expression was inversely associated with CIMP-high status. Moreover, our data suggest that within CIMP-high cancers (but not within CIMP-low/0 cancers), patients with SMO-expressing tumors may expect better cancer-specific survival compared to those with SMO-nonexpression tumors. Given multiple hypotheses testing and the exploratory nature of our interaction analyses, these findings need confirmation by additional independent studies.

Interestingly, we observed a possible inverse association between SMO expression and *BRAF* mutation/*KRAS* wild type in colorectal cancers, independent of other molecular variables. *BRAF* mutation is present in 10–15 % of colorectal cancers and is associated with inferior prognosis.[66–69] Nonetheless, our results need to be confirmed by independent studies.

There are several limitations to our study. First, data on treatment were limited. We speculated that chemotherapy administration did not substantially differ by tumor SMO expression because the data were not available for treating physicians. Nevertheless, our regression analyses were adjusted for tumor, node, metastasis classification system stage, on which treatment decisions are largely based. Second, despite quite high agreement of readings of the two pathologists for SMO immunohistochemistry, there was still a 9 % discordance rate.

In conclusion, our large cohort study demonstrated that SMO expression in colorectal cancer is inversely associated with phosphorylated AKT expression and CTNNB1 nuclear localization. SMO expression in colorectal cancer may interact with tumor CIMP status to affect patient

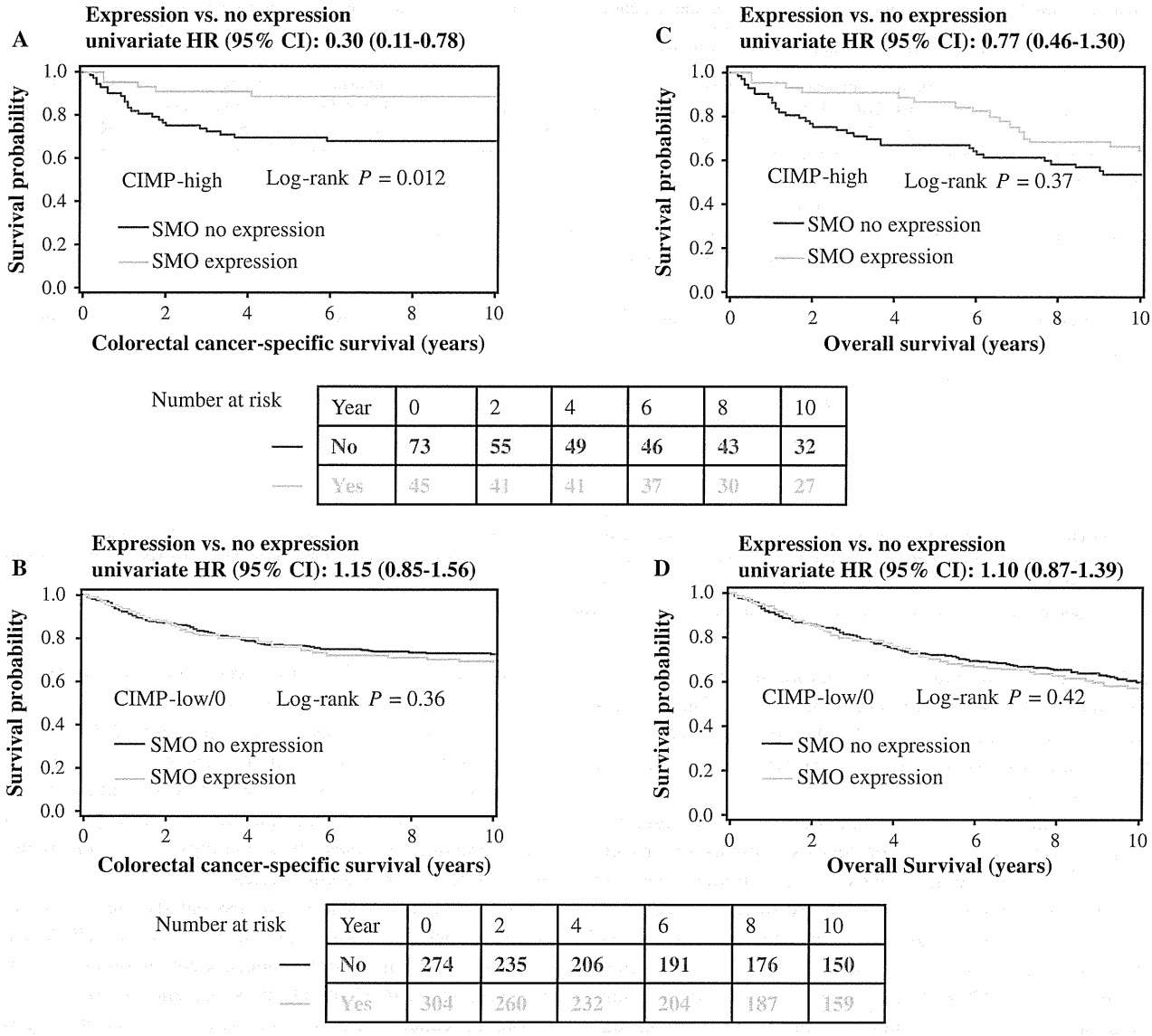

FIG. 2 Colorectal cancer-specific and overall survival in patients with colorectal cancer according to SMO expression status in strata of CIMP status. *CI* confidence interval, *HR* hazard ratio

prognosis, although confirmation by future studies is needed. Our data are compatible with literature supporting a role for SMO in pathway networking in colorectal carcinogenesis.

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