Original Article

DNA Methylation Predicts Recurrence From Resected Stage III Proximal Colon Cancer

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BACKGROUND: In colorectal cancer (CRC), DNA methylation anomalies define distinct subgroups termed CpG island methylator phenotype 1 (CIMP1), CIMP2, and CIMP-negative. The role of this classification in predicting recurrence and disease-free survival (DFS) in resected stage III CRC was evaluated. **METHODS:** Sporadic cancers from 161 patients were analyzed. Bisulfite pyrosequencing was used to examine the methylation of 2 global DNA methylation markers (LINE-1, Alu) and 9 loci (*MINT1, MINT2, MINT31, P16, hMLH1, P14, SFRP1*, SFRP2, and WNT5A). Mutations in BRAF and KRAS were assayed. **RESULTS:** Gene hypermethylation clustered in discrete groups of patients, indicating the presence of CIMP. K-means clustering analysis identified 3 discrete subgroups: CIMP1 (n = 22, 13.7%), associated with proximal location and *BRAF* mutations; CIMP2 (n = 40, 24.8%), associated with *KRAS* mutations; and CIMP-negative (n = 99, 61.5%), associated with distal location. In proximal CRC, CIMP1 was correlated with a higher recurrence rate (53% for CIMP1, 18% for CIMP2, and 26% for CIMP-negative) and a worse DFS ($P = .015$). Also in proximal CRC, LINE-1 methylation was lower in patients whose cancer recurred compared with those whose cancer did not recur ($P = .049$). In multivariate analysis, CIMP1 and low LINE1 methylation were independent prognostic factors for DFS in proximal CRC ($P = .008$ for classification by K-means clustering analysis; $P = .040$ for LINE-1 methylation status). **CONCLUSIONS:** DNA methylation is a useful biomarker of recurrence in resected stage III proximal but not distal CRC. However, as the number of CIMP1 cases was small in distal CRC, further study is required to validate our findings. ***Cancer* 2011;117:1847–54.** *© 2010 American Cancer Society.*

KEYWORDS: colon cancer, CpG island methylator phenotype, LINE-1, methylation biomarker.

Cancer develops under the influence of genetic and epigenetic alternations.[1,2] DNA methylation is a main component of epigenetics, and the relationship between DNA methylation and carcinogenesis has been extensively studied in various cancers.[1,3] In cancer, DNA methylation has 2 main patterns. Cancer cells have decreased global methylation compared with normal cells,[4,5] which may be involved in genetic instability.[6-8] LINE-1 methylation density is a good indicator of global methylation.[9] Decreased LINE-1 methylation was associated with decreased survival in colorectal cancer.[10] In contrast, there is increased CpG Island methylation density in the promoter regions of tumor-suppressor genes.[1,3] This hypermethylation causes decreased gene expression and is involved in carcinogenesis through silencing tumor-suppressor genes.

DNA methylation has been extensively studied in colon cancer.[3,11] Colon cancer can be divided into subsets according to DNA methylation patterns: CpG Island methylator phenotype–positive (CIMP+) and CIMP− groups. CIMP+ cancers show distinct clinicopathologic features including female preponderance, older age, proximal colon location, and mucinous and poorly differentiated histology.[11] They are associated with microsatellite instability (MSI) and *BRAF* mutations.[12] Although, CIMP+ cancers share many clinical features with MSI-related cancers, their prognosis is different. CIMP+ cancers are associated with poor prognosis,[13,14] whereas MSI-related cancers have a good prognosis.[15,16] Multiple genes may be responsible for the prognostic effects of DNA methylation in colorectal cancer (CRC), including *WNT* pathway genes. Abnormal activation of the WNT/beta catenin pathway is frequently found in gastrointestinal cancers[17,18] and colon carcinogenesis.[17,19,20] One of the important mechanisms of WNT pathway activation is the decreased production of gene products that have an inhibitory effect on the WNT pathway. *SFRP, DKK*, and *WNT5A* are recognized to be

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WNT pathway antagonists.[21] The promoter region of *WNT5A* is frequently methylated in colon cancer tissue, and preserved *WNT5A* expression was reported to be associated with a good prognosis in colon cancer,[22] whereas promoter methylation of WNT pathway antagonists was associated with a poor prognosis.[23,24]

There are embryologic, histologic, physiologic, and biochemical differences between proximal colon and distal colons.[25,26] Proximal colon consists of the cecum, ascending colon, and proximal two-thirds of the transverse colon. It develops from the embryonic midgut, whereas the distal colon develops from the embryonic hindgut. There is a difference in gene expression between the proximal and distal colons. More than 1000 genes were found to be differentially expressed in the adult colon but only 87 genes differentially expressed in the fetal stage.[27] Around 70% of these genes were found to be highly expressed in the distal colon. Based on these results, it is reasonable to consider the proximal and distal colons to be physiologically distinct, and the cancers that arise from them could also be quite different.

In this study, we evaluated the potential role of DNA promoter methylation biomarkers, genetic biomarkers (*KRAS* and *BRAF* mutations) and global DNA methylation biomarkers to predict recurrence and disease-free survival (DFS) according to cancer location in curatively resected stage III colon cancer.

MATERIALS AND METHODS

Tissue Samples

We used 161 stage III colon adenocarcinoma specimens obtained at the time of curative resection at the Yonsei Cancer Center, Severance Hospital (Seoul, Korea) from 1997 to 2006. The specimens were immediately frozen in liquid nitrogen and stored at −80°C. We excluded cases of hereditary colon cancer. The collection of samples was approved by the Severance Institutional Review Board, and informed consent was obtained from patients to use their surgical specimens and clinicopathologic data for research purposes.

Assay of DNA Methylation

Bisulfite-treated genomic DNA was used to evaluate the methylation status of 2 global methylation markers (LINE-1, Alu) and the methylation status of 9 CpG islands (*MINT1, MINT2, MINT31, hMLH1, p16, p14, SFRP1, SFRP2*, and *WNT5A*). Bisulfite treatment of DNA was performed with an EpiTect bisulfite kit (Qiagen, Valencia, CA) according to the manufacturer's protocol. One microliter of bisulfite-treated DNA was used as a template in subsequent polymerase chain reaction (PCR). All PCR assays included a denaturation step at 95°C for 30 seconds, followed by an annealing step at various temperatures for 30 seconds, and an extension step at 72°C for 30 seconds. After PCR, the biotinylated strand was captured on streptavidin-coated beads (Amersham Bioscience, Uppsala, Sweden) and incubated with sequencing primers. Pyrosequencing was performed with PSQ HS 96 Gold single-nucleotide polymorphism reagents on a PS QHS 96 pyrosequencing machine (Biotage, Uppsala, Sweden). The protocol for pyrosequencing was described in detail previously.[28] Pyrosequencing quantitatively measures the methylation status of several CpG sites in a given sequence. Therefore, we could determine the mean percentage of methylation of detected sites as a representative value.

Assay of BRAF and KRAS Mutations

Genomic DNA was used to study the mutation status of *BRAF* and *KRAS* genes. Mutation status was determined with pyrosequencing assays. Mutations of *BRAF* codon 600 and *KRAS* codons 12 and 13 were determined by a pyrosequencing machine (Biotage, Uppsala, Sweden).[29,30]

Data Analysis and Statistics

Pyrosequencing presents methylation and mutation levels as a continuous value. The methylation status of CpG Island markers was analyzed as either a continuous or categorical variable (negative, methylation level $<15\%$; positive, methylation level $\geq 15\%$). The methylation status of global methylation markers was analyzed as either continuous or categorical variables (divided into 2 groups by the median value). Mutation status was analyzed as a categorical variable (wild-type status, mutation level $<15\%$; mutation, mutation level $\geq 15\%$). All clinicopathologic variables except age were used as categorical variables. Differences in continuous variables between 2 groups were evaluated by the Student *t* test, and differences in categorical variables were evaluated by the chi-square test. Correlation of methylation level between methylation biomarkers was analyzed by calculating Spearman's nonparametric correlation coefficients (r and P). K-means clustering on the basis of both genetic and epigenetic profiling was performed to identify potential discrete subgroups among colon cancer patients. K-means clustering analysis was conducted using ArrayTrack version 3.4.0

(NCTR/FDA, Jefferson, Ark). DFS was measured from the date of resection of colon cancer to the date of event or the last follow-up date before December 31, 2008. Event was defined as recurrence, death due to any cause, or development of a second primary in colorectal cancer. Median follow-up duration was 46 months. The Kaplan-Meier method was used to calculate and display disease-free survival curves, and the log-rank test was performed to determine differences among all groups. The Cox proportional hazards regression method was used to determine independent prognostic factors.

All P values were 2 sided, and a $P < .05$ was considered statistically significant.

RESULTS

Clinicopathologic Characteristics

We studied 161 patients selected based on sample availability. Mean age was 61 years (range, 31-84 years), 93 patients were male (58%), and 76 tumors (47%) were in the proximal colon. All patients were treated with postoperative adjuvant chemotherapy consisting of 5-fluorouracil and leucovorin. Among the 156 patients with adequate follow-up data, there were 48 recurrences (31%), 23 in the proximal colon and 25 in the distal colon. The clinicopathologic features of the patients analyzed by tumor location are summarized in Table 1. Overall, there were no differences in sex, age, histology, differentiation, T stage, N stage, or recurrence rate between those with proximal colon cancer and those with distal colon cancer.

Table 1. Clinicopathologic Characteristics According to Tumor Location

	Location		
	Proximal	Distal	*P*
Number	76	85	
Age, y mean (range)	61.9 (31-84)	60 (31-82)	.315
Sex			
Male	44	49	.975
Female	32	36	
Histology			
Adeno	70	83	.106
Mucinous	6	2	
Differentiation			
WD	8	10	.824
MD	58	70	
PD	4	3	
T stage			
1	1	2	.664
2	5	10	
3	55	58	
4	15	15	
N stage			
1	54	57	.585
2	22	28	
Recurrence			
No	52	56	.979
Yes	23	25	

WD, well differentiated; MD, moderately differentiated; PD, poorly differentiated; adeno, adenocarcinoma; mucinous, mucinous adenocarcinoma.

Mutations of BRAF, KRAS, and Microsatellites

The mutation status of *BRAF* at codon 600 and of *KRAS* at codon 12,13 was determined by pyrosequencing. There were 7 cases (4.3%) with *BRAF* mutation, all in the proximal colon. As for *KRAS*, 56 cases (34.8%) showed mutations, and these were not associated with age, sex, tumor location, or histology. There was no case harboring both *KRAS* and *BRAF* mutations. Microsatellite instability (MSI) status was previously determined, and data were available on 91 patients. Of these, 17 (18.7%) showed MSI, which was associated with proximal location.

Gene-Specific Methylation Analysis

The methylation frequencies of the 9 genes analyzed were 17% for *MINT1,* 22% for *MINT2,* 19% for *MINT31,* 16% for *P16,* 6% for *MLH1,* 9% for *P14,* 54% for *WNT5A,* 93% for *SFRP2,* and 99% for *SFRP1*. Methylation of all the genes showed significant positive correlations with each other (not shown), consistent with the presence of CIMP in a subset of cases. Seven genes (all except *SFRP1* and *SFRP2*) showed strong correlations and appeared to be excellent CIMP markers. For descriptive purposes, we defined CIMP as positive when 3 or more of the 7 markers were positive. Based on this definition, 29 cases (18%) were CIMP+. As previously reported,[11,12] CIMP was significantly associated with female sex, proximal colon cancer, *BRAF* mutation, and microsatellite instability–high (MSI-H).

K-Means Clustering Based on Combined Genetic and Epigenetic Information

We have previously reported that colon cancer falls into 3 distinct groups based on combined genetic and epigenetic analysis.[30] In the current data set, 3 distinct groups were similarly identified by K-means clustering analysis of the promoter methylation status of the 9 genes and the mutation status of *BRAF* and *KRAS* (Fig. 1). The clinicopathologic and molecular features of the 3 groups are

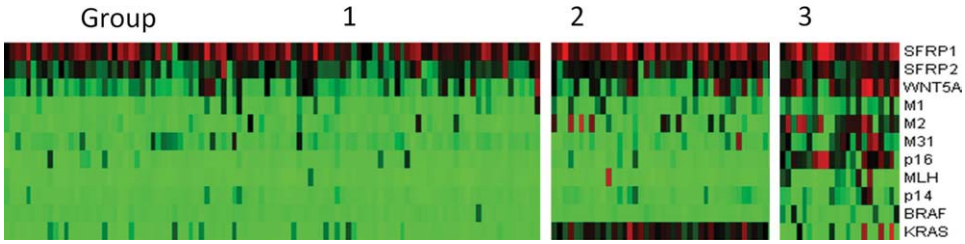

Figure 1. K-means clustering analysis based on genetic and epigenetic information is shown. Ninety-nine cases were classified as group 1 (low methylation), 40 cases were classified as group 2 (intermediate methylation), and 22 cases were classified as group 3 (high methylation).

summarized in Table 2. The CIMP1 (methylation-high) group was characterized by a relatively high rate of *BRAF* mutations and MSI-H, and most cases (82%) were in the proximal colon. The CIMP2 group was characterized by a high rate of *KRAS* mutation, whereas the CIMP-negative group had rare mutations and low levels of methylation. CIMP2 and CIMP-negative cases were slightly more common among the distal cancers.

Given the reproducibility of the classification of colon cancer into 3 groups, we evaluated recurrence rates and DFS by this classification. Overall, CIMP1 cases had the highest rate of recurrence (9 of 21, or 42.9%, compared with 39 of 135, or 28.9%, in CIMP2 and CIMP-negative), but this was not statistically significant. Because of the strong site imbalance in the distribution of cases, we evaluated DFS by site. There was a significant DFS difference among the 3 groups in proximal colon cancer (Fig. 2a), showing the worst survival in CIMP1 (HR, 3.9; 95% CI, 1.08-14.35; $P = .015$). This difference was not observed in distal colon cancer ($P = .304$; Fig. 2b).

CIMP1 is associated with both MLH1 methylation and *BRAF* mutation. Previous data suggested that MLH1 methylation is associated with good prognosis, whereas *BRAF* mutation leads to a poor prognosis.[31] To test this, we subdivided CIMP1 into 3 groups (MLH1 methylation positive, n = 7; MLH1 methylation negative and *BRAF* mutation positive, n = 4; neither MLH1 methylation nor *BRAF* mutation, n = 11) and evaluated DFS. Despite the very small number of patients in each group, there was a significant DFS difference between the 3 subgroups, showing the best survival in MLH methylation+ patients and the worst survival in MLH methylation and *BRAF*-mutated patients ($P = .016$; Fig. 2c).

Global DNA Methylation Analysis

We evaluated global DNA methylation using LINE-1 and Alu methylation. High methylation of LINE-1 was

Table 2. Clinicopathologic Characteristics According to Groups by K-Means Clustering Analysis

	Group			
	1, Low Methyl	2, Intermediate	3, High Methyl	*P*
Number	99	40	22	
Age, y (mean)	60.8	59.3	64.3	.257
Sex				
Male	60	22	11	.608
Female	39	18	11	
Site				
Proximal	42	16	18	.002[a]
Distal	57	24	4	
Histology				
Adeno	94	38	21	.995
Mucinous	5	2	1	
Differentiation				
WD	12	5	1	.646
MD	78	32	18	
PD	4	1	2	
T stage				
1	3	0	0	.344
2	8	5	2	
3	74	26	13	
4	14	9	7	
N stage				
1	70	26	15	.802
2	29	14	7	
Recurrence				
No	67	29	12	.424
Yes	28	11	9	
LINE1, mean±SD	50.1±8.4	50.4±4.6	53.6±6.2	.14
MSI status[b]				
MSS or MSI-L	47	21	6	.034[a]
MSI-H	10	2	5	

WD, well differentiated; MD, moderately differentiated; PD, poorly differentiated; adeno, adenocarcinoma; mucinous, mucinous adenocarcinoma.
[a] Significant difference among groups by K-means clustering analysis.
[b] Available for a subset of patients only.

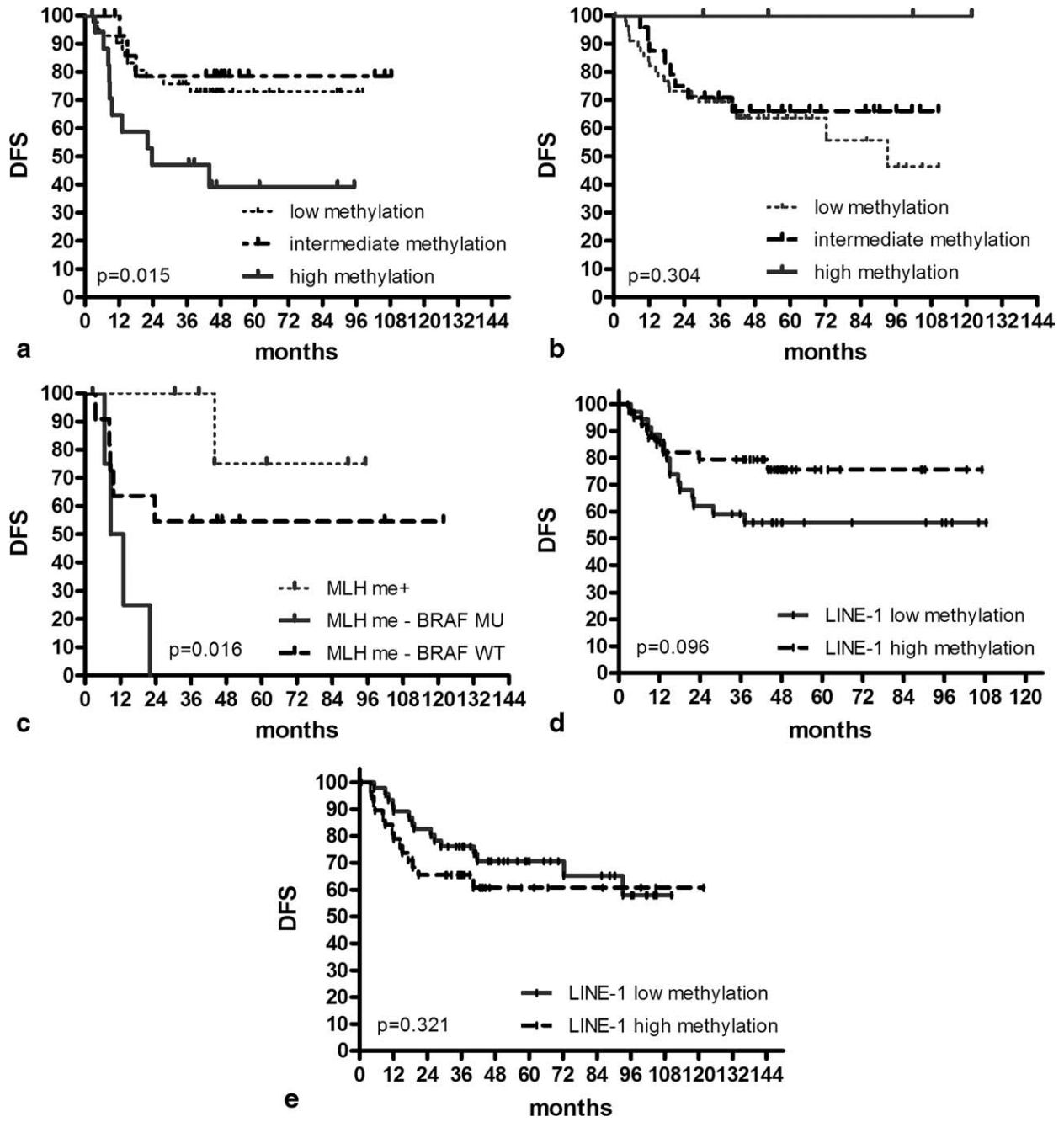

Figure 2. Kaplan-Meier survival estimates in colon cancer are shown. DFS in proximal colon cancer (a) and distal colon cancer (b) according to groups classified by K-means clustering analysis using genetic and epigenetic information is shown. DFS in colon cancer (c) according to groups classified by MLH methylation and BRAF mutation status in the high methylation group is shown. DFS in proximal colon cancer (d) and distal colon cancer (e) according to groups classified by LINE-1 methylation is shown.

significantly associated with CIMP1, high methylation of Alu, low methylation of SFRP1, and low methylation of SFRP2. No differences were noted in age, sex, tumor location, histology, KRAS status, or MSI status according to LINE-1 methylation status. In proximal colon cancer, methylation level of LINE-1 was significantly lower in patients with recurrent cancer than in patients with nonrecurrent cancer (48.5 $\pm$ 8.6 vs 52.3 $\pm$ 7.2, respectively; $P = .049$; Fig. 3). No difference was found in distal colon cancer. Low methylation of LINE-1 in the proximal colon

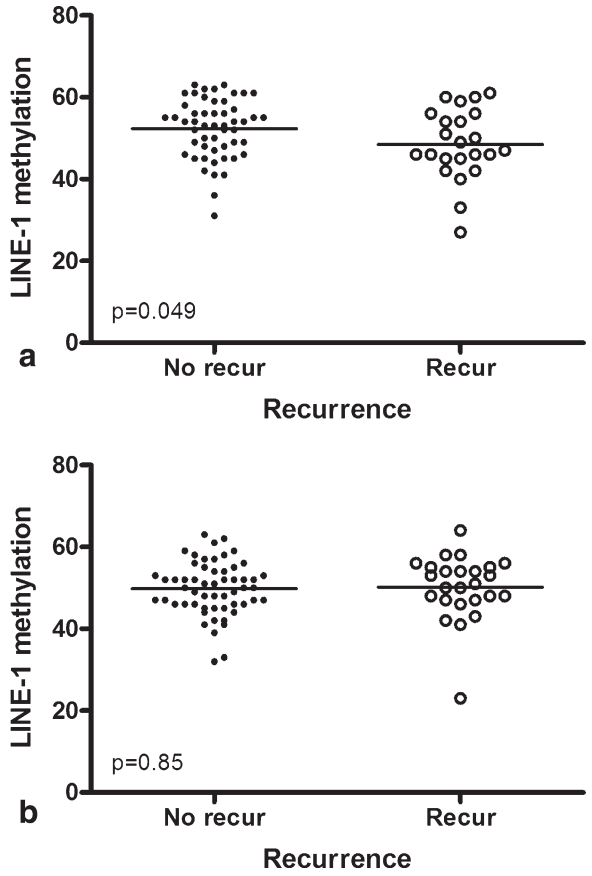

Figure 3. Methylation levels of LINE-1 according to recurrence are shown. In proximal colon cancer, the methylation level of LINE-1 was significantly lower in patients with recurrent cancer than in patients with nonrecurrent cancer (a), but not in patients with distal cancer (b).

cancer was associated with a trend towards shorter DFS ($P = .097$). In distal colon cancer, there was no difference in DFS survival according to methylation status of LINE-1 (Fig. 2d,e).

Multivariate Analysis of DFS

Table 3 shows multivariate analysis that included known clinicopathologic characteristics associated with DFS (T stage, N stage, tumor location, CEA) and other potential molecular biomarkers (LINE-1 methylation status, *BRAF* status, *KRAS* status, and grouping by K-means clustering analysis of genetic and epigenetic information). LINE-1 methylation and grouping by K-means clustering analysis were independent prognostic factors for DFS in proximal colon cancer ($P = .040$ for LINE-1 methylation status; $P = .008$ for grouping by K-means clustering analysis). N stage was the only independent prognostic factor in distal colon cancer ($P = .004$). In multivariate analysis using

Table 3. Multivariate Cox Regression Model of Prognostic Factors of DFS in Colon Cancer Patients According to Location

	HR	95% CI	*P*
All colon			
N stage			.001
N1	1	1.48-4.43	
N2	2.56		
Proximal colon			
LINE-1 methylation			.040
High	1	1.04-5.64	
Low	2.43		
K-means clustering			.008
Group 1	1.28	0.36-4.59	
Group 2	1	1.22-16.25	
Group 3	4.44		
Distal colon			
N stage			.004
N1	1	1.43-6.35	
N2	3.01		

HR, hazard ratio; CI, confidence interval.

classification based on epigenetic information alone (CIMP) instead of using K-means clusters, *BRAF* status, LINE-1 methylation status, and CIMP status were independent prognostic factors for DFS in proximal colon cancer ($P = .035$ for *BRAF* status; $P = .007$ for LINE-1 methylation status; $P = .011$ for CIMP status) but not in distal colon cancer.

DISCUSSION

In this study, we showed that methylation biomarkers play a differential role in CRC recurrence and DFS by tumor location. Using gene methylation and mutation, we could cluster cases into 3 distinct groups. The high methylation group demonstrated a significant association with CIMP+, *BRAF* mutation, and MSI-H. The intermediate methylation group showed a significant association with high frequency of *KRAS* mutation. These molecular features were almost the same as those that we previously reported[30] in a different population of patients, demonstrating the reproducibility of this classification. Methylation-related biomarkers influenced recurrence and DFS in resected stage III proximal colon cancer but not in distal colon cancer. The study adds to the growing literature on differences between proximal and distal cancers and suggests that these may have to be taken into account in the management of and clinical trials in this disease.

CIMP-negative colon cancers are evenly distributed throughout the colon, but CIMP1 colon cancers are principally located in the proximal colon.[32] The cause of this

difference is unknown. It may be that site-specific carcinogens, or differences in the cell of origin may explain this variation in DNA methylation. Still, only about half of proximal cancers are CIMP1, and our data now suggest that DNA methylation may help in classification of these patients for prognostic purposes. A high risk for recurrence of stage III CRC may lead to more intensive surveillance and novel adjuvant therapy strategies. We have previously reported that the methylation status of several CIMP markers was associated with poor survival in stage IV CRC,[14] but it is not yet known whether this is modulated by site. One of the paradoxical findings on the effects of CIMP on survival is the finding that CIMP1 is also associated with MLH1 methylation, which results in MSI. MSI is generally a favorable prognostic factor in CRC.[15,16] MSI was relatively rare in the population of patients we studied, which explains why the dominant effect of CIMP was negative on DFS. In a small pilot analysis, we did find that MLH1 methylated cases had a good outcome, whereas CIMP1, MLH1 unmethylated cases had a strikingly high recurrence rate, regardless of *BRAF* mutation. It is interesting to consider why the 2 groups of CIMP1 cases (MLH1 methylated/unmethylated) would have such opposing consequences. An attractive hypothesis is that the poor prognosis is imparted by DNA hypermethylation of genes such as *WNT5A* that results in more aggressive behavior (increased invasion for example). In turn, this invasive phenotype may be countered by induction of an immune response that is most pronounced in MSI-positive cases.[33]

Here, we confirm a previous report on the prognostic impact of LINE1 methylation on the outcome of CRC[10] but show that this is also limited to proximal cancers. Others have examined the effect of global DNA methylation on clinical outcomes in various cancers. In 1 study, the level of global DNA methylation was significantly lower in prostate cancer than in normal prostate, but there was no difference according to recurrence.[34] In ovarian cancer, the level of LINE-1 methylation was significantly lower than that in normal tissue, and there was a shortened survival in the low methylation group.[35] The mechanisms by which a low level of LINE-1 methylation is associated with poor outcome remain to be determined. Possibilities include association with genomic instability or with activation of expression of selected genes.

It is interesting to consider why DNA methylation was associated with recurrence in proximal cancers but not in distal cancers. A simple possibility relates to the rarity of CIMP1 cases in distal cancer, which limited our power to detect a prognostic impact there. Larger studies should address this issue. It is also possible that some other molecular marker, not measured here, has a dominant effect on recurrence and thus negates the effect of methylation differences on outcomes. Indeed, deletions of chromosome 18 are associated with recurrences in stage III CRC,[36] and these are more common in distal cancers and CIMP-negative cases.[37,38] Thus, it may be that DNA methylation is a dominant prognostic factor in proximal cancers, whereas genetic instability is a dominant prognostic factor in distal cancers. Our studies and these hypotheses, which need to be confirmed in a larger population, pave the way for individualized management of stage III CRC.

In summary, methylation biomarkers such as methylation of WNT5A, CIMP markers, and LINE-1 can predict disease recurrence and DFS in resected stage III proximal colon cancer but not in distal cancer. However, as the number of CIMP1 cases of distal CRC in our study was small, further study is required to validate our findings. Classification of CRC by both genetic and epigenetic profiles will likely improve the capability of predicting prognosis and of applying tailored therapy in this disease, but this classification will also have to take into account differences between proximal and distal cancers.

CONFLICT OF INTEREST DISCLOSURES

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