The American Journal of Pathology, Vol. 177, No. 5, November 2010
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DOI: 10.2353/ajpath.2010.091103

Gastrointestinal, Hepatobiliary and Pancreatic Pathology

Development of Sporadic Microsatellite Instability in Colorectal Tumors Involves Hypermethylation at Methylated-In-Tumor Loci in Adenoma

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Microsatellite instability (MSI) and genomic hypermethylation of methylated-in-tumor (MINT) loci are both strong prognostic indicators in a subgroup of patients with sporadic colorectal cancer (CRC). The present study was designed to determine whether the methylation of MINT loci during the progression of adenoma to CRC is related to MSI in CRC cases. Methylation index (MI) was measured by absolute quantitative assessment of methylated alleles at seven MINT loci in primary CRC with contiguous adenomatous and normal tissues of 79 patients. Results were then validated in primary CRC tissues from an independent group of 54 patients. Increased MI of both MINT loci 1 and 31 was significantly associated with MSI in CRC and was specific for adenoma. Total MI and the number of methylated loci were threefold (*P* = 0.02) and fivefold (*P* = 0.004) higher, respectively, in adenomas associated with microsatellite-stable CRC versus microsatellite-unstable CRC. MINT MI was found to be correlated with mismatch repair protein expression, MSI, *BRAF* (V600E) mutation status, mut-L homologue 1 methylation status, and disease-specific survival in the second independent validation group of patients. MI of specific MINT loci may be prognostic indicators of colorectal adenomas that will develop into sporadic microsatellite-unstable CRCs. Increased MINT locus methylation appears to precede MSI and may have utility in defining clinical pathology in the absence of features of malignant invasive tumors. *(Am J Pathol 2010, 177:2347–2356; DOI: 10.2353/ajpath.2010.091103)*

Epigenetic changes in epithelial cells, such as DNA methylation of CpG islands, have been related to the genesis and progression of some gastrointestinal cancers.[1,2] Aberrations in DNA methylation are considered to be as important as genetic alterations in gastrointestinal tumor initiation and progression. In colorectal cancer (CRC), both hypomethylation and hypermethylation of promoter-region related CpG islands have been correlated with clinical and histopathological parameters.[3,4] A number of tumor-related gene promoter regions are methylated in premalignant dysplastic lesions such as hyperplastic polyps,[5] aberrant crypt foci,[6,7] and adenomas.[8–10] Furthermore, progressive genomic and epigenomic aberrations may be linked in CRC progression.[11] For instance, it has been reported that widely increased methylation in sporadic CRCs overlaps with microsatellite instability (MSI).[12–15] Studies to pinpoint the onset of MSI have been reported in hereditary nonpolyposis colorectal cancers that carry germline mutations (mt) in mismatch repair (MMR) genes,[13,14,16,17] but as of yet, no study has examined this process in sporadic CRC.

Spontaneous regression of colorectal polyps is known to occur. Therefore, a critical issue in molecular analysis of colorectal precursor tumor lesions is whether a lesion with low or intermediate adenomatous dyplasia would develop into an invasive cancer. The DNA extracted from such lesions may not contain key premalignant aberrations. We recently described an on-slide sodium bisulfite

Supported in part by the Leslie and Susan Gonda (Goldschmied) Foundation (Los Angeles, CA) and Ruth and Martin H. Weil Fund (Los Angeles, CA).

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Accepted for publication July 13, 2010.

None of the authors disclosed any relevant financial relationships.

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modification (SBM) technique for gene methylation analysis in small (1 to 2 mm^2) tissue areas isolated from a single section of paraffin-embedded archival tissue (PEAT).[18] On-slide SBM allows comparison of gene methylation in the primary CRC, the contiguous adenoma lesion, and normal epithelium when these three tissue types are present on the same tissue section. Simultaneous assessment of methylation and MSI changes in CRC, adenoma, and normal tissues from the same patient using the on-slide SBM technique would provide an accurate analysis model to test development of these epigenetic and genetic events during CRC formation.

In a previous study, our group demonstrated the technical feasibility of using methylated-in-tumor (MINT) loci 1, 2, 12, and 31 to detect methylation differences between areas of the same colorectal tissue section.[8] MINT loci are conservative human genomic sequences that adhere to the CpG island definition.[19,20] They are found in noncoding genomic regions, and their gene regulatory purpose or other functional attributes are unknown. We have previously shown the clinical utility of methylation levels of specific MINT loci in rectal cancer[20] and melanoma.[4] Other groups have studied MINT locus methylation in colorectal[20–22] and gastric cancers.[20–24] Methylation of MINT loci has also been linked to MSI in CRC.[25,26] However, whether this was present in the precursor adenoma is an important question that our analysis model may answer.

The objective was to determine whether MINT locus hypermethylation is associated with MMR during early stages of CRC development. Methylation at MINT loci was quantified by using the on-slide SBM technique combined with absolute quantitative assessment of methylated alleles (AQAMA). We further evaluated MSI status, *KRAS* mt at codons 12 and 13, *BRAF* V600E mt, and methylation status of the *MutL homolog 1* MMR gene (*MLH1*) in primary CRC as well as the contiguous precursor lesion to rigorously assess whether these events occur at early stages of CRC development. Our hypothesis was that MINT locus methylation, *MLH1* promoter region methylation, and MSI in adenoma tissue are higher in those adenomas adjacent to MSI-high (MSI-H) compared with microsatellite-stable (MSS) invasive CRC.

Materials and Methods

Patient Specimens

For the first phase of the study, we identified patients whose resected CRC specimen contained histopathologically confirmed areas of adenoma as well as invasive cancer in our cancer registry database. Excluded were any cases without available PEAT specimens. Consecutive patients were identified in reverse chronological order until an adequate sample size was reached. The final cohort of 115 patients who underwent surgical resection of CRC between 1996 and 2009 at the Saint John's Health Center was selected for the first phase of the study.

A single H&E section was prepared and mounted; 7-μm sections were consecutively cut and mounted on silane-coated glass slides for DNA studies. Areas of adenoma (serrated and nonserrated), carcinoma, and normal tissue as well as the adenoma type were identified by a surgical pathologist (R.R.T.) with expertise in CRC.[8,27]

To further validate the findings of the first phase of the study, PEAT blocks of operative specimens were obtained from 54 patients who underwent surgical resection of CRC at the Leiden University Medical Center (LUMC) between 1990 and 2001. All specimens had been previously analyzed for MMR sufficiency at LUMC's pathology department. From each PEAT block, a single section was cut for H&E staining, and 7-μm sections were consecutively cut on coated slides for on-slide SBM. Tumor areas were identified and marked by an expert CRC pathologist (H.M.). Study protocols for assessment of patient specimens were approved by the institutional review boards at the LUMC and at Saint John's Health Center.

AQAMA and MLH1 Methylation Assessment

DNA from PEAT was modified *in situ* by sodium bisulfite according to our previously reported protocol.[18] AQAMA of MINT loci 1, 2, 3, 12, 17, 25, and 31 was performed, and data were analyzed in a manner as previously described.[8,28] *MLH1* methylation status was analyzed by capillary-array-electrophoresis methylation-specific PCR.[27,29,30] Primer sequences were selected based on previous literature and correlation with MLH1 protein expression determined.[31–35]

MSI, KRAS, and BRAF mt Analysis

For MSI assessment in the first phase of the study, a tissue section from each of the specimens was deparaffinized and stained with hematoxylin to identify adenoma and CRC cells for DNA isolation, as previously described.[8] Normal epithelial cells were harvested from a separate tissue block of the same specimen (ie, from noninvolved resection margins). As biomarkers for MSI assessment, we used three quasi-monomorphic mononucleotide repeats (BAT25, BAT26, and BAT40) and two microsatellite, dinucleotide repeats (D2S123 and D5S346) found in the revised Bethesda guidelines to interrogate the specimens.[36] Forward primers were dye-labeled for automated high-throughput multiplex detection by capillary array electrophoresis[37] (CEQ 8000XL; Beckman Coulter, Fullerton, CA). Forward primer sequences for BAT25, BAT26, BAT40, D2S123, and D5S346 were as follows: 5′-CCTCGCCTCCAAGAATGTAA-3′, 5′-GCAGTCAGAGCCCTTAACCTT-3′, 5′-AAGATTAACTTCCTACACCACAACC-3′, 5′-TGGCCAGAGAA ATTAGACACA-3′, and 5′-TTCAGGGAATTGAGAGTTACAGG-3′, respectively; corresponding reverse primer sequences were as follows: 5′-TGCTTTTGGTTACCACACTTCA-3′, 5′-CCATTTAAAGCTAGTTATCTAATCCA-3′, 5′-GTAGAGCAAGACCACCTTGT-3′, 5′-TCT GACTTGGATACCATCTATCTATCT-3′ and 5′-TCACTCTAGTG-

ATAAATCGGGAAA-3′. Differences in PCR product fragment length among different tissue categories were visualized by the CEQ software (Beckman Coulter). PCR products from the five amplified microsatellite regions in adenoma and cancer were compared with the reference normal epithelium. *KRAS* (codons 12 and 13) and *BRAF* (V600E) mt were assessed by a peptide nucleic acid clamp-based quantitative real-time PCR assay as previously described.[38,39] These assays were performed in triplicate and carried out at least twice to confirm accuracy. A gene was considered mutated when the results were uniformly positive in the triplicates under the optimal conditions. Respective normal and positive PEATs as well as cell line controls were included in each assay.

MMR and mt Status Analysis

The diagnostic techniques used by the LUMC's pathology department for MSI status and MMR protein expression assessment are described previously.[40] Briefly, MSI status was assessed by using MSI Analysis System (Promega Corp., Madison, WI; five mononucleotide and two pentanucleotide repeats).[41] Immunostaining of MMR proteins was performed with anti-MLH1 (clone G168-728; 1:50; BD Biosciences, Franklin Lakes, NJ) and anti-PMS2 (clone A16-4; 1:50; BD Biosciences). Immunohistochemistry (IHC) staining was performed on PEAT sections (4 μm in thickness) from tissue microarray (TMA). IHC staining patterns of these MMR proteins were evaluated by using normal epithelial, stromal, or inflammatory cells, or the centers of lymphoid follicles as internal controls, as previously published.[42] TMAs contained three cores punched from each primary tumor. Individual cores of the TMA were scored as either positive (showing nuclear staining in at least some tumor cells) or negative. Cases were considered positive if at least one TMA tissue core showed nuclear staining; if not, cases were considered negative. Cases in which both tumor and internal control stained negative were not included in the study. Cases were scored by two independent reviewers (H.M. and N.F.C.C. de M.); in case of a discrepancy, both reviewers reassessed the slides for consensus. *KRAS*[43] and *BRAF*[44] mt were detected by means of sequencing as previously described.

Biostatistical Analyses

Significance of changes in methylation index (MI) at individual MINT loci and total MI was evaluated with nonparametric tests for related and independent sample sets (Wilcoxon's rank-sum test; Mann-Whitney's *U*-test). Fisher's exact test (two-tailed) was used to assess significance of intergroup differences in the prevalence of *MLH1* methylation, *BRAF*, or *KRAS* mt.

Spearman's rank correlation coefficient between quantitative MINT MI and *MLH1* MI was assessed as a nonparametric measure of correlation. Correlations with clinical parameters were tested with Pearson's χ^2 test and, for ordinal variables, with Mann-Whitney's *U*-test or Kruskal-Wallis' test. Postoperative distant recurrence probability and disease-free and overall survival were estimated with Kaplan-Meier plots, and significance was assessed with the log-rank test. Cox regression models considered the following variables that were entered in a stepwise manner: age, nodal stage, MSI status, and tumor differentiation. $P < 0.05$ (two-sided) was considered significant. SPSS (SPSS Inc., Chicago, IL) statistical software version 16.0.1 was used for all analyses.

Results

MINT Locus Methylation and MSI during CRC Development

In the first phase of the study, we assessed operative specimens from 115 patients who underwent open resection of CRC and whose specimen, according to the pathology report, contained adenoma as well as cancer (see Table 1 for patient characteristics). Fifty of these specimens included normal tissue. Twenty-seven of the 115 patients were excluded from the study because the adenomas contained high-grade dysplasia and/or had carcinoma without evidence of submucosal invasion. Of the remaining 88 specimens that contained low- or medium-grade adenomatous dysplasia, 79 still had invasive carcinoma tissue after reviewing the cut sections. MSI status was analyzed in normal and cancer tissue of the 79 patients by using five established genomic markers.[36] Specimens from nine patients (11%) showed instability in ≥4 biomarkers; these patients comprised the MSI-H group. Specimens from 65 patients (83%) did not show a shift in any of the markers, and specimens from five (6%) patients had a single dinucleotide repeat affected; these 70 patients with ≤1 aberrant MSI marker comprised the MSS group. The median age of MSI-H patients was 81.4 years (range, 69 to 91). Figures 1 and 2 show representative PCR product analyses of AQAMA and capillary array electrophoresis.

The MI of normal, adenoma, and cancer tissue was determined for each MINT locus of patients in MSI-H and MSS groups (Figure 3). Methylation levels of MINT loci 1, 2, 3, 12, and 31 were significantly higher in CRC than in normal epithelium. Methylation levels of MINT loci 1, 2, 12, 17, and 31 were significantly higher in MSI-H CRC compared with MSS CRC. MINT17 MI was MSI-related in CRC but was also present in normal tissue. Methylation levels of MINT loci 1 and 31 were significantly higher in adenomas contiguous to MSI-H CRC than in adenomas contiguous to MSS CRC. The average number of tumor-specific, MSI-related MINT loci showing MI >0.1 was 1.5 (SD ± 1.4) in adenomas from specimens containing MSI-H CRC, whereas on average only 0.3 (SD ± 0.6) MINT loci had MI >0.1 in adenomas from specimens containing MSS CRC ($P = 0.004$; Figure 4B). The total MI of MINT loci 1, 2, 12, and 31 was 4.5 times higher in adenomatous tissue from specimens containing MSI-H CRC ($P = 0.02$; Figure 4A). MSI status was significantly correlated to right-sided tumor location and not to serrated adenoma type (Table 1). MI was significantly higher in serrated versus nonserrated adenomas only at MINT1

Table 1. Patient Characteristics

Characteristics	Total patient group (*n* = 115)	Patients with cancer tissues (*n* = 79)	Microsatellite unstable (*n* = 9)	Microsatellite stable (*n* = 70)	*P*
Sex, *n* (%)					
Male	45 (39)	35 (44)	3 (33)	33 (47)	0.44
Female	70 (61)	44 (56)	6 (66)	37 (53)	
Age, year					
Mean (SE)	76.1 (1.2)	75.0 (1.0)	81.4 (7.3)	76.1 (10.6)	0.15
Tumor location, *n* (%)					
Cecum	24 (21)	16 (20)	3 (33)	14 (20)	0.03
Colon ascendens	15 (13)	9 (11)	4 (44)	5 (7)	
Hepatic flexure	6 (5)	4 (5)	0	4 (6)	
Colon transversum	17 (15)	12 (13)	2 (22)	9 (13)	
Colon descendens	4 (4)	3 (4)	0	3 (4)	
Sigmoid colon	13 (11)	9 (11)	0	8 (11)	
Rectosigmoid	12 (10)	9 (10)	0	9 (13)	
Rectum	24 (21)	17 (22)	0	18 (26)	
Adenoma type, *n* (%)					
Serrated	9 (8)	5 (6)	2 (22)	3 (4)	0.10*
Classic	106 (92)	74 (94)	7 (88)	67 (96)	
Villoglandular	7 (6)	4 (5)	0	4 (6)	
Tubular	11 (10)	8 (10)	2 (22)	6 (9)	
Tubulovillous	15 (13)	10 (13)	1 (11)	9 (13)	
Villous	73 (63)	52 (66)	4 (44)	48 (68)	

*Fisher's exact test evaluating MSI status in serrated versus classic adenoma in patients with adenoma as well as carcinoma available for analysis (*n* = 72).

and MINT31 ($P = 0.002$ and $P = 0.02$, respectively). There were no significant differences in MINT methylation level of cancer tissues contiguous to serrated versus classic adenoma. Methylation levels were relatively low in the two serrated/MSI-H adenomas compared with the nonserrated/MSI-H adenomas.

From this we conclude that increased methylation of MINT loci 1, 2, 12, 17, and 31 in adenoma correlates with MSI-H sporadic CRC, and increased methylation of MINT loci 1, 2, 12, and 31 is specific for MSI-H CRC. Increased methylation of MINT 1 and 31 was adenoma-/CRC-/MSI-specific, and these loci were differentially methylated in serrated versus nonserrated adenomas. The adenomas associated with MSI-H CRC with high MINT loci methylation did not show any distinct histopathology features. Results indicate that total MI and number of affected MSI-specific MINT loci are indicators of colorectal adenomas that will become microsatellite-unstable CRC.

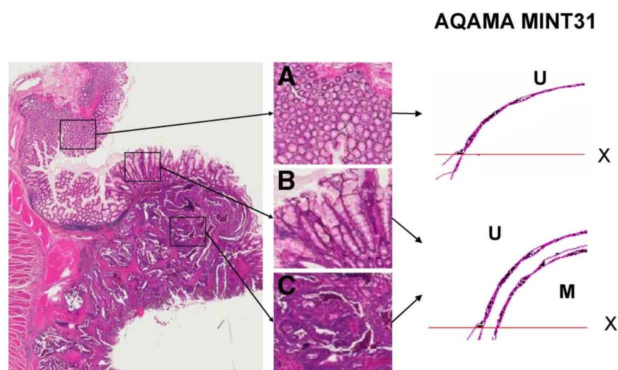

Figure 1. Representative molecular analyses in normal (**A**), adenoma (**B**), and cancer (**C**) tissues. AQAMA (MINT31) real-time PCR results showing results in triplicate of exponential amplification of methylated (M) and unmethylated (U) dye probe signals. X; PCR threshold cut-off. The vertical axis represents signal intensity, horizontal axis represents PCR cycle number.

MLH1 Methylation Status and MSI during CRC Development

We subsequently determined whether the correlation between MINT methylation levels and MSI in colorectal adenomas could be explained by methylation of the *MLH1* MMR gene. The relation between *MLH1* methylation status and MSI was assessed in a case-control approach. Normal, adenomatous, and cancer tissues were acquired from all nine patients in the MSI-H group and from 13 randomly selected patients with sufficient available tissue in the MSS control group. This case-control approach was opted for because after multiple sectioning of the PEAT blocks, some cases lost either their cancer or their adenoma tissue. There were no significant differences between cases and controls with respect to age, sex, and polyp histopathology type. *MLH1* was methylated in all cancers from the MSI-H group compared with two cancers from the MSS group ($P < 0.001$). *MLH1* was methylated in 6 of 9 MSI-H adenomas compared with 2 of 13 adenomas paired to MSS cancers ($P = 0.02$). Only three of six (50%) MSI-H adenomas with *MLH1* methylation showed to be MSI-H. In combination, MI of MINT1, 2, 12, and 31 with collateral methylation of *MLH1* constitute a specific biomarker panel of adenomatous colorectal tissue that shows MSI or will develop into a sporadic MSI-H CRC. The results further indicate that MINT locus methylation along with *MLH1* methylation precedes MSI and appears before histopathology signs of CRC development.

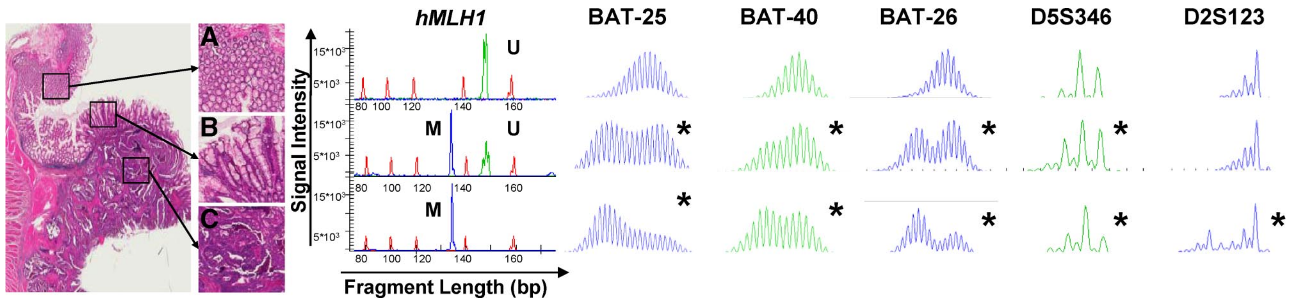

Figure 2. Representative molecular analyses in normal (**A**), adenoma (**B**), and cancer (**C**) tissues. Capillary array electrophoresis results of amplified dye-labeled PCR products of five MSI biomarkers and *MLH1* methylation-specific (M, in blue) and unmethylated-specific sequence (U, in green) primer sets. Red peaks represent the DNA ladder signal. The horizontal axis represents size of the PCR product in bp. The vertical axis represents arbitrary units of signal intensity of detected amplicons. **Asterisk** indicates MSI positive.

BRAF *and* KRAS *mt during CRC Development*

Studies in CRC have shown correlations between increased methylation of important tumor-related genes and mt of *BRAF* (V600E) and *KRAS* (codons 12 and 13), often in combination with MSI-H.[15,19,45,46] The significance is not clearly understood; however, it has been suggested that these events synergistically induce a high-risk phenotype that results in a clinically distinct subtype of CRC. We determined *BRAF* mt and *KRAS* mt in the same group analyzed for *MLH1* methylation status analysis (9 MSI-H cases and 13 MSS cases); the results are summarized in Table 2. *BRAF* mt status was significantly correlated to MSI and increased MINT MI. *KRAS* mt was more frequent in MSS adenomas and carcinomas, although this did not reach statistical significance. These results were as expected based on previous reports. This assessment demonstrated that genetic and epigenetic events synergize in the earliest phase of CRC development.

Figure 3. Box plots summarizing MI values for each MINT locus in specimens containing normal, adenoma, and cancer tissues from patients in MSS and MSI-H groups.

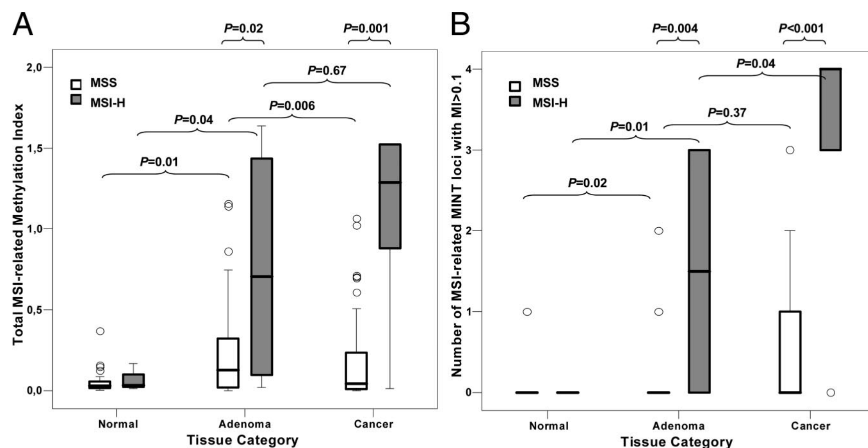

Figure 4. Box plots of MI data for specimens containing normal, adenoma, and cancer tissues from patients in MSS and MSI-H groups. **A:** *y* axis represents total MI of four MSI-related MINT loci (MINT1, 2, 12, and 31). **B:** *y* axis represents the number of MSI-related MINT loci with MI greater than 0.1.

Quantitative MINT Locus Methylation and Mismatch Repair

Methylation levels of five different MINT loci were related to methylation of *MLH1*, *BRAF* mt status, and to MSI in CRC specimens from 79 patients in the first phase of the study. These findings were corroborated by using an independent cohort of 54 patients with CRC whose *MLH1* and PMS2 expression as well as MSI status were known. We first corroborated whether increased MINT MI linearly correlates to *MLH1* MI, and subsequently whether it affects MMR at the protein level. MIs at the MSI-associated MINT loci (1, 2, 12, 17, and 31) and *MLH1* MI were measured in 54 CRC specimens that were previously analyzed for MMR sufficiency. Two outcome parameters were analyzed: total MI (defined by the sum of MIs of MINT loci 1, 2, 12, 17, and 31) and number of methylated MINT loci. The number of methylated MINT loci in a specimen was determined as the number of MINT loci (1, 2, 12, 17, and 31) that exceeded the MSI-H related MI cut-off established during the first phase of the study (average MI $\pm$ 1 SD in MSS CRC specimens). Correlation analysis showed that *MLH1* MI was closely associated with total MI ($\rho = 0.43$; $P = 0.002$) and with the number of methylated MINT loci ($\rho = 0.400$; $P < 0.001$). Table 3 shows the association between MLH1 protein expression and MI. Epigenomic down-regulation of *MLH1* was demonstrated by the lower expression of its co-protein PMS2 in all cases.[47] Examples of IHC and capillary-array-electrophoresis methylation-specific PCR results are given in Figure 5. Subsequently, all MMR protein-deficient cases showed MSI by PCR analysis. Again we analyzed two outcome parameters: number of affected MINT loci and total MI. These results demonstrated a linear correlation between quantitative MINT methylation, *MLH1* down-regulation, and subsequent MMR deficiency, and showed the importance of the number of involved loci as well as total MI.

BRAF mt and *KRAS* mt were also assessed in this patient group. *BRAF* mt cases ($n = 11$, 20%) were significantly associated with MSI-H ($P = 0.003$) and underexpression of *MLH1* ($P = 0.003$). *KRAS* mt tumors ($n = 14$, 26%) did not show any significant associations with mismatch repair parameters. *BRAF* mt tumors had significantly increased methylation at MINT1, 2, 12, 17, and 31 ($P < 0.001$, $P < 0.001$, $P = 0.001$, $P < 0.001$, and $P = 0.003$, respectively). None of the *BRAF* mt tumors had *KRAS* mt. Methylation levels did not differ significantly between *KRAS* mt tumors and *KRAS* wild-type tumors.

Clinical outcome in our validation group was analyzed with respect to the number of MINT loci that showed MSI-related methylation. The result (Figure 6) confirmed previous reports of a direct correlation between methylation levels and recurrence-free survival. Multivariate analysis showed that the number of methylated, MSI-related MINT loci was an independent predictor of distant

Table 2. Correlation of *KRAS* mt, *BRAF* mt, Tissue Histopathology, Microsatellite Instability, and MINT Methylation Index

	Carcinoma ($n = 22$)			Adenoma ($n = 21$)			Carcinoma ($n = 22$)			Adenoma ($n = 21$)		
Mutation status	BRAF mt+	BRAF mt−	*P*	BRAF mt+	BRAF mt−	*P*	KRAS mt+	KRAS mt−	*P*	KRAS mt+	KRAS mt−	*P*
Case-control group, *n* (%)	4 (18)	18 (82)		5 (24)	16 (76)		10 (45)	12 (55)		8 (38)	13 (62)	
MSS group ($N = 13$), *n* (%)	0	13 (100)	0.02	0	13 (100)	0.003	8 (62)	5 (38)	0.10	7 (54%)	6 (46)	0.09
MSI-H group ($N = 9$), *n* (%)	4 (44)	5 (56)		5 (62)†	3 (38)†		2 (22)	7 (78)		1 (13)†	7 (87)†	
Total MINT* MI, mean (SEM)	1.9 (0.6)	0.5 (0.2)	0.009	1.4 (0.6)	0.3 (0.1)	0.03	0.6 (0.4)	0.9 (0.3)	0.39	0.2 (0.1)	0.8 (0.3)	0.16
MINTs* with MI >0.1, mean (SEM)	3.8 (0.3)	1.2 (0.4)	0.02	2.6 (0.9)	0.8 (0.2)	0.09	1.0 (0.5)	2.3 (0.6)	0.09	0.8 (0.3)	1.6 (0.5)	0.34

*MINT1, 2, 12, and 31.
†$n = 8$ for MSI-H adenoma cases.

Table 3. Relation of Methylated Loci and MI to *MLH1* Methylation Status and Protein Expression

No. of MINT loci methylated	No. of patients	*MLH1* Average methylation index (SEM)	*P**	*MLH1* IHC Nonexpressing† (%)	*P**
0	32	0.14 (0.22)	0.007*	5 (16)	0.002*
1	7	0.08 (0.10)		2 (29)	
2	4	0.01 (0.01)		1 (25)	
3	5	0.72 (0.34)		1 (20)	
4	2	0.42 (0.25)		2 (100)	
5	4	0.43 (0.37)		4 (100)	

*Kruskal-Wallis test for correlation between methylated MINT loci and *MLH1* MI or *MLH1* protein expression.
†Confirmed by underexpression of PMS2.

recurrence-free survival (hazard ratio: 0.20; 95% confidence interval: 0.04 to 0.96; $P = 0.02$), disease-free survival (hazard ratio: 0.33; 95% confidence interval: 0.12 to 0.87; $P = 0.04$), and overall survival (hazard ratio: 0.38; 95% confidence interval: 0.14 to 1.00; $P = 0.05$), along with nodal stage and patient age.

Discussion

Our study adds important findings on genomic and epigenomic events at an early stage of CRC development. Several groups have identified epigenetic silencing of *MLH1* as the cause for MSI in nonhereditary CRC.[13–15,48] By using a unique assay system, we demonstrated a reproducible correlation between MSI, *BRAF* mt, *MLH1*, and MINT locus methylation between CRC and its associated precursor adenoma. This concurrent analysis of contiguous invasive and noninvasive tissue is preferable to analysis of adenoma tissue from "premalignant" polyps collected during colonoscopy, because it is known that not all polyp-associated adenomas will progress to CRC. Genetic and/or epigenetic aberrations in the adenoma tissue with contiguous cancer that we analyzed may have progressed compared with adenoma tissue from polyps

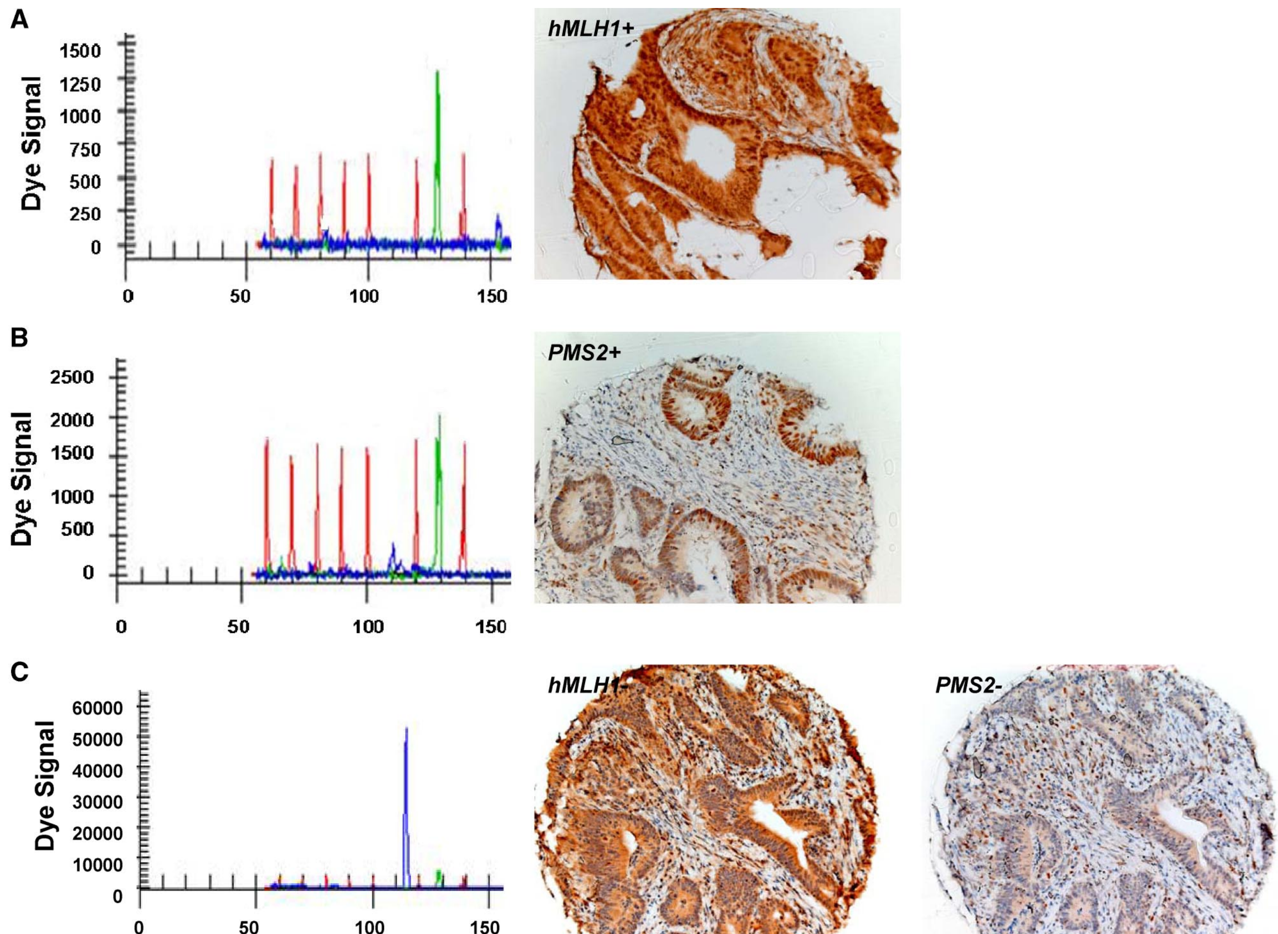

Figure 5. Representative IHC staining of MLH1 and PMS2 proteins. **A** and **B:** *MLH1*-expressing CRC and PMS2-expressing CRC, respectively, with their corresponding capillary-array-electrophoresis methylation-specific PCR results for *MLH1* showing unmethylated signals only. **C:** CRC showing methylation of the *MLH1* promoter region with corresponding absence of nuclear staining of *MLH1*, which is confirmed by underexpression of PMS2.

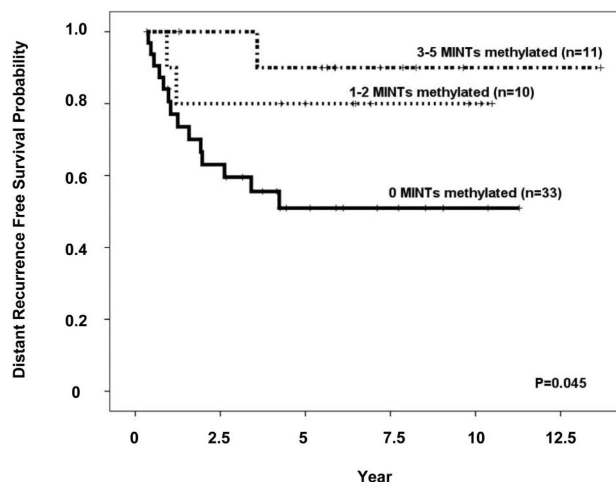

Figure 6. Kaplan-Meier curves for distant recurrence probability in patients stratified by 0, 1 to 2, or 3 to 5 MINT loci with MSI-related methylation.

without invasive component. The histopathological stage, however, was premalignant, and the main point of our study is that MINT markers may identify histopathological negative lesions that are likely to become MSI-H CRCs.

In this study methylation levels were measured for seven genomic MINT loci related to cancer and located on different chromosomes. The MINT biomarkers were originally detected in a study by Toyota et al.[49] These investigators used a genome-wide screening method to show differential methylation in DNA from a colon cancer cell line (Caco2) compared with normal epithelium. We used MINT loci as biomarkers in this study, as we have previously shown that quantitative assessment of MINT biomarkers can distinguish between early CRC and benign disease.[8] It is unclear whether MINT loci are representative of genomic hypermethylation. However, they have been well associated with the CpG island methylator phenotype (CIMP) in CRC. There is currently no consensus on CIMP biomarkers such as the Bethesda guidelines for MSI assays. Clinically relevant correlations between methylation markers, MSI, and *BRAF* mt/*KRAS* mt in CRC have been reported by using different techniques and combinations of three,[50] eight,[51] or five[52,53] different methylation biomarkers. Our results show new application of MINT biomarkers to identify precursor lesions of MSI-H CRC disease and further demonstrate significant associations with CIMP-features (*BRAF* mt, *MHL1* methylation, MSI, and disease survival).

In adenomas associated with MSI-H CRCs, there was significantly more hypermethylation of *MLH1*. This suggests that CRC precursor lesions with a higher MI at MINT loci may be more likely to have methylation of the *MLH1* MMR gene. This has not been reported to date. The relationship between degree of methylation and MSI was further validated at the MMR protein level in an independent patient group. MINT MI linearly correlated with *MLH1* MI, and the underexpression of MLH1 protein and MSI were demonstrated in the more methylated CRCs. This suggests that sporadic MSI is due to deregulation of a CRC's MMR system, which may be a direct result of *MLH1* epigenetic gene silencing in a tumor with increased MINT methylation. The level of DNA methylation appears to be an underlying mechanism of sporadic MSI, and the accumulation of DNA methylation affects the MMR system early during CRC development. We further corroborated the validity of the quantitative MINT methylation AQAMA assay by reproducing its previously reported prognostic value in colorectal and gastric cancer.[51,53,54]

Recent studies on DNA hypermethylation in CRC include *BRAF* mt and *KRAS* mt analyses. *BRAF* mt is more frequent in sporadic CRCs showing MSI-H (approximately 50%), CIMP and *MLH1* methylation,[55,56] and is possibly associated with shorter survival.[51] Specific single-nucleotide mt of *KRAS* have predictive value in stage III/IV CRC, likely related to systemic treatment response.[57–59] However, as of yet, no study has demonstrated a functional relation between MMR impairment and reduced cell signaling properties of *KRAS* mt or *BRAF* mt containing CRCs. In both the first-phase analysis and the validation patient groups, we could not show significant correlations between KRAS mt status, MSI, and MINT methylation. Because colon cancer has a >40% to 60% frequency of *KRAS* mt, it is likely that there is no strong relation to MSI. Two recent, large studies could not either identify a correlation between *KRAS* and MSI in colon cancer.[60,61] Our results do confirm the association between *BRAF* mt and MINT locus hypermethylation, and add that these events occur early in the development of CRC in nonserrated adenomas with low or intermediate dysplasia.

This study demonstrated that AQAMA of MINT loci may have clinicopathologic utility for early identification of patients at risk for developing MSI-H phenotype sporadic cancers. A potentially useful approach would be to examine MINT locus methylation in polyps collected during routine colonoscopies; changes in MINT methylation over time might correlate with a high risk for CRC development. Patients whose adenomas have high MINT locus methylation may benefit from scheduling more frequent screening colonoscopies. Our results further show that MINT loci methylation levels can discriminate normal from adenomatous tissue. MINT biomarkers could be explored to be used as a fecal DNA screening tool to identify patients who should undergo colonoscopy. MSI-H precursor lesions may be identified by MINT biomarkers 1, 2, 12, and 31, and MSS lesions may be identified by MINT3 methylation. Furthermore, it is known that MSI-H cancers respond differentially to common chemotherapeutics. For instance, irinotecan is suggested to be more effective than 5-Fluro-uracil in these tumors. In the development of new targeted drugs, preventive treatment regimens may be available for high-risk patients with adenomas that show high MINT locus methylation. In addition, there is increasing evidence for MSI-induced generation of novel tumor-specific carboxy-terminal frameshift peptides in MSI-H cancers.[62] Polyps with high MINT locus methylation may become a stratification factor in trials of frameshift peptide-based vaccines.

This is the first major study to demonstrate a correlation between MSI and MINT hypermethylation in CRC precursor lesions, supporting that MMR may be dampened by

progressive epigenetic events. The degree of genomic hypermethylation in CRC precursors may be a potential cause of sporadic MSI CRC. Specific MINT locus methylation may have utility for early identification of colorectal polyps with high likelihood of developing into sporadic MSI-H cancers.

Acknowledgments

We thank Linhda Nguyen for technical assistance and Kana Rivera for editorial assistance.

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