

Morphological and Molecular Heterogeneity within Nonmicrosatellite Instability-High Colorectal Cancer¹

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Abstract

Colorectal cancer (CRC) has traditionally been classified into two groups: microsatellite stable/low-level instability (MSS/MSI-L) and high-level MSI (MSI-H) groups on the basis of multiple molecular and clinicopathologic criteria. Using methylated in tumor (MINT) markers 1, 2, 12, and 31, we stratified 77 primary CRCs into three groups: MINT++ (>2), MINT+ (1–2), and MINT– (0 markers methylated). The MSS/MSI-L/MINT++ group was indistinguishable from the MSI-H/MINT++ group with respect to methylation of *p16^{INK4a}*, *p14^{ARF}*, and *RIZ1*, and multiple morphological features. The only significant difference between MSI-H and non-MSI-H MINT++ cancers was the higher frequency of *K-ras* mutation ($P < 0.004$) and lower frequency of *hMLH1* methylation ($P < 0.001$) in the latter. These data demonstrate that the separation of CRC into two nonoverlapping groups (MSI-H versus MSS/MSI-L) is a misleading oversimplification.

Introduction

A series of CRCs³ may be perceived as a biological continuum within which there may be discontinuities as revealed by particular tests. A test may form the basis of a classification if it generates groups that are distinguished by clinical, pathological, or molecular features. Stratification on the basis of testing for DNA MSI identifies a MSI-H subset with well-established clinical and pathological correlates (1, 2). Silencing of the DNA mismatch repair gene *hMLH1* by methylation of a CpG island within its promoter region is the usual mechanism for generating sporadic MSI-H CRC (3–5). The fact that no single feature is the exclusive property of MSI-H CRC indicates that the disease is a continuum rather than an aggregation of separate disease types.

Testing CRCs with DNA microsatellite markers identifies a low-level group (MSI-L) that is not as well defined as the MSI-H subset. Most CRCs reveal MSI-L to some degree when tested with a large panel of microsatellite markers, but the distribution of instability is nonrandom. At one end of the spectrum is an excess of “super-stable” cancers, whereas at the other end there is an excess of “true MSI-L” cancers with between 10 and 25% of markers showing instability (6).

One explanation for the variation in MSI-L levels is somatic alteration of genes having a relatively subtle influence on the efficiency of DNA mismatch repair. In keeping with this suggestion, methylation and loss of expression of the DNA repair gene *MGMT* is associated with MSI-L status (7).

An alternative but related approach to classifying CRC is through testing for the presence of methylation of CpG islands. Sporadic MSI-H cancers with methylation of *hMLH1* show the CIMP in which multiple CpG-rich genes and MINT loci are methylated (8). However, CIMP-positive cancers include some that are non-MSI-H (9). Because methylation is a specific mechanism for gene silencing, whereas MSI is merely an epiphenomenon serving as a biomarker for DNA mismatch repair deficiency, it might be supposed that methylator status would serve as the more valid basis for tumor classification. Classification on the basis of MSI testing might result in the artificial splitting of a group defined on the basis of CIMP status. To explore this hypothesis we have examined the distribution of multiple variables across CRCs stratified by both MSI and CIMP status. The study shows that cancers with multiple MSI-H-related features occur outside the MSI-H group, a finding that may be relevant to theories of tumorigenesis.

Materials and Methods

The material was derived from a prospectively collected, hospital-based frozen tumor bank comprising 879 CRC specimens, and classified as MSI-H, MSI-L, and MSS using at least six DNA microsatellite markers (10, 11). Instability in $\geq 40\%$ of markers was taken as the breakpoint distinguishing MSI-H from MSI-L cancers. Specimens comprising 16 MSI-H, 38 MSI-L, and 23 MSS cancers were included in the study on the basis of prior molecular characterization with regard to microsatellite status, *K-ras* mutation, methylation of *MGMT*, and immunohistochemical evaluation of *MGMT* and *hMLH1* expression, as well as availability of DNA for additional methylation assays (7, 12). The MSS cases were a randomly selected subset of this dominant group.

Methylation-specific PCR was used to test for methylation as has been described previously for *p16^{INK4a}* (13), *p14^{ARF}* (14), *MGMT* (15), and *RIZ1* (16).

COBRA for *hMLH1* methylation (17) was modified to include nested primers for increased sensitivity. The nested sense primer was 5'-GATTTAG-TAATTTATAGAGT-3' and antisense was 5'-AATACCTTCAACCAAT-CAC-3'. The primary product was amplified in a total volume of 25 μ l containing 2.5 μ l bisulfite-modified DNA, 1 \times PCR buffer (Applied Biosystems), 0.2 mM deoxynucleoside triphosphate, 1.3 mM Mg²⁺, 4 μ M of each primer, and 0.5 units Red Hot Taq (Applied Biosystems). One μ l of the primary product was reamplified in a total volume of 50 μ l using the same reagent concentrations. Fifteen μ l of the nested PCR product was digested with 0.8 units *RsaI* at 37°C overnight. Digested samples were analyzed on 10% polyacrylamide gels and visualized with ethidium bromide. MINTs 1, 2, 12, and 31 were assayed by COBRA (Fig. 1).⁴

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³ The abbreviations used are: CRC, colorectal cancer; MSS, microsatellite stable; MSI-L, low-level microsatellite instability; MSI-H, high-level microsatellite instability; *MGMT*, *O*-6-methylguanine-DNA-methyltransferase; CIMP, CpG island methylator phenotype; MINT, methylated in tumor; COBRA, combined bisulfite restriction analysis.

⁴ Internet address for detailed protocols: <http://www.med.jhu.edu/methylation/primers.htm>.

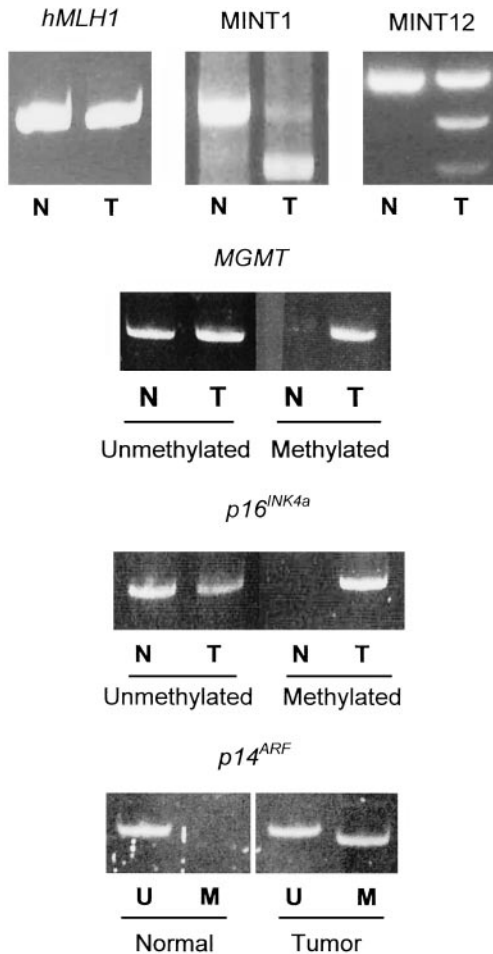


Fig. 1. Methylation analysis by COBRA for *hMLH1*, *MINT1*, and *MINT12*, and by methylation-specific PCR for *MGMT*, *p16^{INK4a}*, and *p14^{ARF}*. Histology for this case is in Fig. 2.

Morphological features were scored without knowledge of either MSI or methylator status: (1) grade (poor or other); (2) cytoplasm (eosinophilic or basophilic); (3) morphological heterogeneity with respect to either tumor type or grade of tumor differentiation (absent or present); (4) tumor infiltrating (intraepithelial) lymphocytes (absent or present); (5) invasion (expanding or infiltrating); (6) nuclear chromatin pattern (condensed or vesicular); (7) nuclear shape (round or ovoid); (8) nucleolus (large or small); (9) extracellular mucin (absent or present); (10) solid or medullary pattern (absent or present); (11) goblet cells (absent or present); and (12) epithelial serration (absent or present).

Cancers were grouped as MINT++ (3–4 markers methylated), MINT++ (1–2 markers methylated), and MINT–. All 16 of the MSI-H cancers were MINT++ (group A, $n = 16$). Non-MINT++/MSI-H cancers may arise through germ line or somatic mutation of a DNA mismatch repair gene. There were 6 MSI-H cancers that showed little or no methylation of MINT loci. These cancers were excluded from this study on the basis that they may have been derived from patients with hereditary nonpolyposis colorectal carcinoma or represent examples of somatic mutation of *hMLH1*. The non-MSI-H cancers were grouped as B (MINT++; $n = 8$), C (MINT++; $n = 29$), and D (MINT–; $n = 24$).

Morphological and genotypic characteristics of tumors were compared across levels of methylation and MSI using Pearson's χ^2 test for association and Fisher's Exact test (for rare features). As the morphological characteristics of tumors are related, we also conducted multivariate analysis to determine factors that were independently associated with methylation. In particular, anatomical location, tumor heterogeneity, presence of tumor infiltrating lymphocytes, invasion, and extracellular mucin were interrelated, so were adjusted for in all of the analyses. When comparing the four methylation/MSI groups,

multinomial logistic regression was used (18), and when comparing two methylation groups, binary logistic regression was conducted. All of the analyses were performed using SAS for Windows release 8.2. A $P < 0.05$ was used to determine statistical significance.

Results

Groups A and B (MINT++) were combined and compared with group C plus D (MINT++/-) with respect to morphological features. Crudely, features significantly associated with MINT++ cancers were: tumor heterogeneity ($P < 0.001$), tumor-infiltrating lymphocytes ($P = 0.004$), round nuclei ($P = 0.015$), vesicular nuclei ($P = 0.007$), prominent nucleoli ($P = 0.012$), extracellular mucin ($P < 0.001$), and proximal location ($P < 0.001$). After multivariate analyses, the only features to retain a significant association with MINT++ cancers were extracellular mucin ($P = 0.001$) and proximal location ($P = 0.05$). Group A and B cancers differed from each other only with respect to tumor heterogeneity ($P = 0.04$). Additionally group B cancers were more likely to show diffuse infiltration ($P = 0.03$). There were no differences between group A and group B cancers with respect to methylation of *p16^{INK4a}*, *p14^{ARF}*, or *RIZ1*, but *K-ras* mutation was associated with group B cancers ($P = 0.004$), and more group B cancers showed *MGMT* methylation, although the difference was not significant ($P = 0.12$). Methylation of *hMLH1* occurred in all of the group A cancers and in no group B cancers ($P < 0.001$). There was full immunohistochemical concordance.

After adjusting for confounding factors, group B cancers differed from group C cancers in showing significantly more cases with *p16^{INK4a}* ($P = 0.03$) and *RIZ1* ($P < 0.02$) methylation, and included more cancers with extracellular mucin ($P = 0.05$). There were more group B cancers with methylation of *p14^{ARF}*, although this did not reach significance ($P = 0.09$). Differences between group B and group D cancers were in a similar direction with respect to methylation, although the findings for *p16^{INK4a}* ($P = 0.09$), *p14^{ARF}* ($P = 0.06$), and *RIZ1* ($P = 0.1$) fell short of significance (Table 1). The distribution of specific MINT methylation appeared to be non-random between the four groups. MINT1 methylation was more frequent in group A than group B ($P = 0.02$), whereas MINT31 was more frequently methylated in group B than group A, although the difference was not significant ($P = 0.14$). There was no difference in MINT1 methylation between group B and group C ($P = 0.2$; see Table 1 for crude and adjusted P s across the four groups).

A solid or medullary architecture and an expanding growth pattern are associated with MSI-H cancers. Although these features were not significantly more frequent in group A and B cancers in this study, they were added to heterogeneity, lymphocytes, nuclear shape, chromatin, nucleoli, and mucin to give an overall morphology score (maximum 8). Fifteen of 16 group A cancers scored 5 or more. Four group B (50%), 8 group C (28%), and 4 group D (17%) cancers scored ≥ 5 . Among the 16 non-MSI-H cancers with an MSI-H morphology score of ≥ 5 , 12 showed methylation of target genes other than *hMLH1*. The frequency of methylation of *MGMT*, *p16^{INK4a}*, *p14^{ARF}*, and *RIZ1* in these cases was 56, 19, 31, and 6%, respectively. The 16 cases showing morphological mimicry of sporadic MSI-H cancer presented at a mean age of 72 years, 8 (50%) occurred in the proximal colon, 9 subjects (56%) were female, and 9 (56%) cancers were MSI-L. Figs. 1 and 2 illustrate an MSS, group B cancer showing morphological and molecular mimicry of MSI-H cancers.

Discussion

CRCs may be considered as a continuum in which particular tests such as MSI and CIMP status reveal discontinuities. Features additional to MSI and CIMP status that were tested in this study included

Table 1 Distribution of molecular variables in groups A–D (with percentages in brackets)

	Group A	Group B	Group C	Group D	Crude <i>P</i>	Adjusted <i>P</i> ^a
	MSI-H	non-MSI-H	non-MSI-H	non-MSI-H		
	MINT++	MINT++	MINT++	MINT–		
Total no. patients	16	8	29	24		
MSI-H	16	—	—	—		
MSI-L	—	7	18	13		
MSS	—	1	11	11		
Proximal location	14 (88)	4 (50)	12 (41)	4 (17)	<0.0001	0.05
Poor differentiation	4 (25)	3 (38)	8 (28)	5 (21)	0.80	0.70
Eosinophillic cytoplasm	4 (25)	2 (25)	15 (52)	8 (33)	0.26	0.44
Tumor heterogeneity	11 (69)	2 (25)	3 (10)	6 (25)	<0.0001	0.06
Tumor-infiltrating lymphocyte present	11 (69)	3 (38)	8 (28)	5 (21)	0.013	0.27
Invasion (expanding)	16 (100)	5 (63)	21 (72)	18 (75)	0.06	0.33
Vesicular nucleus	10 (63)	4 (50)	9 (31)	5 (21)	0.04	0.73
Round nucleus	14 (88)	4 (50)	16 (55)	8 (33)	0.008	0.05
Conspicuous nucleoli	10 (63)	4 (50)	10 (34)	5 (21)	0.05	0.47
EC mucin present	15 (94)	5 (63)	8 (28)	19 (79)	<0.001	0.02
Solid pattern present	3 (19)	3 (38)	4 (14)	2 (8)	0.24	0.26
Goblet cells present	5 (31)	4 (50)	9 (31)	6 (25)	0.63	0.42
Serration present	7 (44)	3 (38)	15 (52)	6 (25)	0.27	0.06
K- <i>ras</i> mutation	0 (0)	4 (50)	13 (45)	8 (33)	0.002	0.59
<i>RIZ1</i> methylation	6 (38)	2 (25)	0 (0)	1 (4)	<0.0001	+ ^b
<i>MGMT</i> methylation	3 (19)	4 (50)	15 (52)	10 (42)	0.17	0.11
<i>p16</i> methylation	10 (63)	4 (50)	4 (14)	2 (8)	0.0005	0.06
<i>p14</i> methylation	10 (63)	5 (63)	6 (21)	6 (25)	0.009	0.20
<i>hMLH1</i> methylation	16 (100)	0 (0)	2 (7)	0 (0)	<0.0001	+ ^b
MINT1 methylation	16 (100)	5 (63)	11 (38)	0 (0)		
MINT2 methylation	16 (100)	8 (100)	16 (55)	0 (0)		
MINT12 methylation	15 (94)	8 (100)	11 (38)	0 (0)		
MINT31 methylation	9 (56)	7 (88)	1 (3)	0 (0)		

^a *P*s adjusted for anatomical location, heterogeneity, tumor-infiltrating lymphocyte present, invasion, and extracellular mucin.

^b +, Not included in multivariate analysis because of 0 cells.

12 morphological variables, methylation of *hMLH1*, *p16^{INK4a}*, *p14^{ARF}*, *RIZ1*, and *MGMT*, and K-*ras* mutation. There was a decreasing gradient of MSI-H-associated morphological and molecular features among non-MSI-H cancers stratified as MINT++ (group B), MINT++ (group C), and MINT– (group D; Table 1). Group B cancers showed few morphological or molecular differences from group A cancers. Whereas none showed *hMLH1* methylation, 7 of the 8 were MSI-L. Even the single MSS MINT++ cancer revealed marked morphological (Fig. 2) and molecular (methylation of *p16^{INK4a}*, *p14^{ARF}*, and *MGMT*) overlap with MSI-H cancers. The findings suggest that MSI-H morphology, and *p16^{INK4a}*, *p14^{ARF}*, and

MGMT methylation are not dependent on MSI status. It is more likely that the shared features among MSI-H and subsets of non-MSI-H cancers are explained by a common pathway of tumorigenesis in which methylation plays a key role in gene silencing.

A distinguishing feature of group A and group B cancers (apart from methylation of *hMLH1*) was the high frequency of K-*ras* mutation within the latter. A low frequency of K-*ras* mutation in sporadic MSI-H cancer is described in the literature (11, 19, 20). Within the non-MSI-H group there is evidence that the highest frequency of K-*ras* mutation occurs within CIMP-positive CRCs (9, 21), MSI-L CRCs (11, 22, 23), or both. The highest frequency of K-*ras* mutation

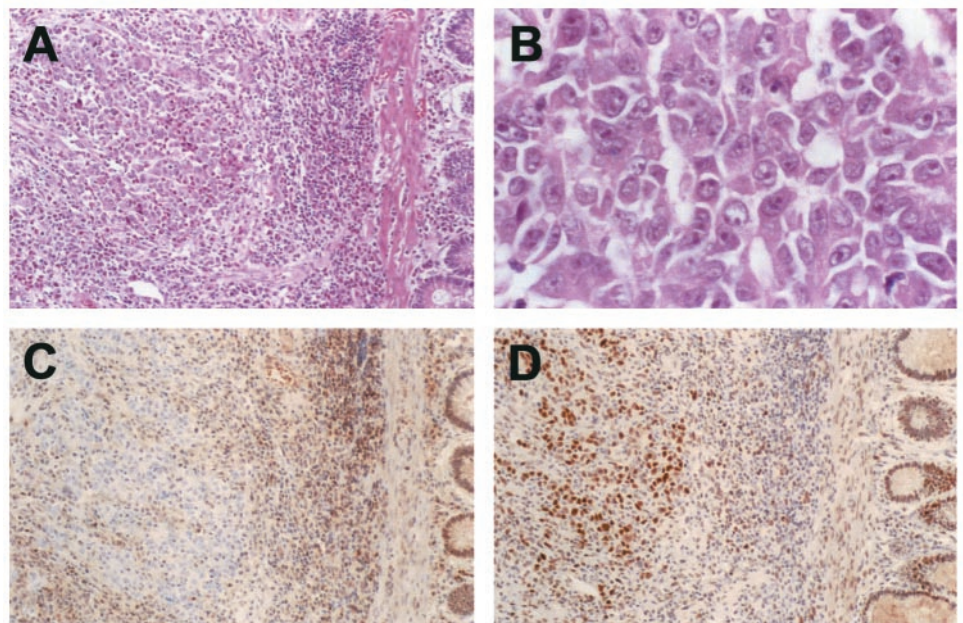


Fig. 2. Section of an MSS/MINT++ cancer (Group B). There was methylation of *p16^{INK4a}*, *p14^{ARF}*, and *MGMT*, but not *hMLH1* (see Fig. 1). The cancer shows a medullary or solid pattern, and is infiltrated by lymphocytes (A). Round, vesicular nuclei with large nucleoli are seen at high magnification (B). Immunohistochemistry revealed loss of expression of MGMT (C), whereas *hMLH1* expression was retained (D).

(90%) is recorded in non-MSI-H CIMP-positive cancers (9), whereas the lowest frequency is recorded in a super-stable group of cancers (6). The declining gradient of *K-ras* mutation in groups B (50%), C (45%), and D (33%) in the present study is consistent with the preceding findings. Methylation of *MGMT* could explain the observations, at least in part. Silencing of this DNA repair gene by methylation of its promoter region has been associated with G to A transition mutation in *K-ras* (15) and with MSI-L status in CRC (7).

The serrated pathway, implying an origin within hyperplastic polyps, admixed polyps, or serrated adenomas, would serve as the basis for the preceding observations. There is evidence that methylation is an important mechanism for gene silencing in this pathway. Serrated polyps probably arise within nondysplastic microscopic lesions called aberrant crypt foci. Both aberrant crypt foci and serrated polyps show molecular heterogeneity with subsets characterized by methylation of *hMLH1* (24, 25) and/or *MGMT* (7, 24), and/or *K-ras* mutation (24). An association between *MGMT* methylation and *K-ras* mutation has also been observed in serrated adenomas (26). It is also relevant that the cytological and architectural features of CIMP+ cancers are also shared by serrated polyps (12). The likeness is functional as well as morphological, extending to the up-regulation of gastric (*MUC5AC*) and intestinal (*MUC2*) secretory mucin in hyperplastic polyps, serrated adenomas, and sporadic MSI-H CRC (27). In this study, glandular serration occurred least frequently in nonmethylated cancers, although the adjusted *P* fell just short of significance (Table 1).

Methylation of *MGMT* showed no association with MINT status. It is possible that cancers in different molecular groups may share a common origin, for example driven by silencing of *MGMT*, but then diverge at an early stage into different tumorigenic pathways. The implication of this suggestion is that a significant proportion of CRCs may originate in microscopic lesions that are nonadenomatous and are initiated by mechanisms other than *APC* mutation, for example methylation of DNA repair genes and/or *K-ras* mutation. *APC* mutation may occur in this alternative pathway but not necessarily as the first event. In summary, a subset of non-MSI-H cancers mimics MSI-H cancers morphologically and with respect to frequent methylation of target genes other than *hMLH1*. Methylation of these genes may result in a selective growth advantage, and in the case of *MGMT* the conferred advantage may be such that methylation of this DNA repair gene occurs in the absence of extensive MINT methylation.

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