Distinct BRAF (V600E) and KRAS Mutations in High Microsatellite Instability Sporadic Colorectal Cancer in African Americans

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Abstract

Purpose: Colorectal cancer develops through genetic, epigenetic, and environmental events that result in uncontrolled cell proliferation. Colorectal cancer incidence and mortality is higher in African Americans (AA) than in the general population. Here, we carried out a molecular analysis of sporadic colorectal cancer tumors from AAs to investigate possible explanations for the observed disparities.

Experimental Design: A total of 222 AA colorectal cancer tumors were analyzed for microsatellite instability (MSI) for protein expression of two DNA mismatch repair genes, MLH1 and MSH2, by immunohistochemistry; for the methylation silencing of *MLH1, p16, APC*, and *APC2* promoters by methylation-specific PCR; and for point mutations in two oncogenes, *KRAS* and *BRAF*, by sequencing.

Results: In our sample, 19.8% of the AAs colorectal cancer tumors were MSI high (MSI-H) and did not associate with any of the clinicopathologic features, except tumor differentiation. Higher levels of inactive DNA mismatch repair proteins MLH1 (41%) and MSH2 (33%) were found by immunohistochemistry. Methylation-specific PCR analysis revealed a high level of methylation for MLH1 (66%), APC (53%), and APC2 (90%), but not for p16 (26%). *BRAF* mutations were only within the MSI-H tumors, whereas most (64%) of *KRAS* mutations were found within the non – MSI-H group.

Conclusions: *MLH1, MSH2*, and *BRAF* alterations are significantly associated with MSI-H phenotype, unlike *APC, APC2* and *KRAS* alterations. The prominent role of DNA mismatch repair gene suppression in MSI-H and a distinctive role of BRAF and KRAS mutations with respect to MSI status are supported by this study.

Colon cancer remains the most common gastrointestinal cancer in the United States, despite recent improvements in the diagnosis and treatment of the disease (1). The incidence and mortality rate of colorectal cancer within African Americans (AA) are higher than in the U.S. general population (1, 2). Many epidemiologic and genetic investigations have focused on AA (2–4) with the goal of deciphering the reasons for such disparities. Whereas one cannot discount socioeconomic reasons for such differences, such as a more advanced stage of disease at diagnosis in AA, other factors also contribute to the progression of colon cancer (5). Dietary and environmental factors play an important role, as shown with Japanese immigrants to the United States who show higher colon cancer incidence than Japanese people living in Japan. The latter also started showing more colon cancer than gastric cancer along with the westernization of their diets (6, 7). The approval by the Food and Drug Administration of Bidil, a drug for heart failure in AAs, illustrates how ethnic/racial genetic specificities can lead to different interactions with a given drug or molecule (8). Colon cancer develops through different pathways, all involving changes at the chromosomal or gene levels. Other modifications occur epigenetically and affect the level of expression of certain targeted genes that are essential for the normal control of cell division within the colon mucosa (9). It is now widely accepted that sporadic colorectal cancers frequently arise from preneoplastic lesions through the activation of oncogenes (*KRAS* and *BRAF*) and the inactivation of tumor suppressor genes (*APC*, *p16*, *p53*, and *DCC*) and mismatch repair genes such as *MLH1* and *MSH2* and, to a lower extent, *PMS2* and *hMSH6* (10, 11). In addition, activating mutations in *BRAF*, a member of the *RAF* gene family, which encode kinases that are regulated by *KRAS* and mediate cellular responses to growth signals, were found to be associated with high-frequency microsatellite instability (MSI-H) cancers (12). The DNA mismatch repair process can be impeded by genes that are either mutated (13) or silenced (4). Such mutations and/or silencing leads to the expression of nonfunctional proteins or to the lack of expression of such genes, respectively. This pathway is primarily responsible for colon cancer development in families with hereditary nonpolyposis colon cancer, which represents ~5% (14) of the

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Translational Relevance

African Americans (AA) are a group at high risk of developing colorectal cancer. The analysis of the molecular parameters that might play a role in the generation of such disparity is of great clinical importance. The results of our genetic and epigenetic study will help the investigators who treat AA patients with colorectal cancer to consider the distinct role of BRAF and KRAS mutations with regard to microsatellite instability status in the process of treatment. In addition, the introduction of silencing of *APC2* genes in AA colorectal cancer patients should provide another new marker involved in colorectal cancer and how it might affect colorectal cancer carcinogenesis.

generally inventoried colon cancer cases. APC2 is a homologue of APC involved in familial adenomatous polyposis colorectal cancer, of which silencing through methylation has been recognized in colorectal cancer (15, 16).

In the present study, we analyzed a large sample of AA colorectal cancer patients for microsatellite instability and BRAF and KRAS mutations, and we correlated the findings with the expression of genes involved in DNA mismatch repair. We also checked the methylation profile of critical promoters that are relevant to colon carcinogenesis: *APC*, *APC2*, *p16*, and *MLH1*. We evaluated and compared the demographic and clinicopathologic data in relation to the generated molecular data to improve our understanding of colon tumorigenesis in AAs.

Materials and Methods

Patients. A total of 222 AA patients (from Howard University and Johns Hopkins Hospitals between 1997 and 2007) who underwent surgical resection for colorectal cancer were included in this study. Formalin-fixed paraffin-embedded archival tissues were collected (with approval from the Institutional Review Boards of the above sites) and clinical data were obtained (including age, site of primary tumor, mucin production, stage, and tumor differentiation). All patients underwent surgery within the given collection period for this study. In addition, patients self-identified as being AAs.

DNA isolation and MSI analysis. Archived and fresh tumor blocks were cut into 5-μm sections on Superfrost slides (Fisher Scientific). The tumor and normal area were diagnosed by a pathologist using the H&E-matched slide and microdissected to pinpoint the tumor areas as well as normal areas from at least two slides. DNA extraction was done by using either a pinpoint DNA extraction kit (Zymo Research) for the archival samples or a Gentra DNA extraction kit (Gentra) for the fresh samples, following the recommendations from respective manufacturer. The extracted tumor and normal matched DNA were used as template in PCR reactions where five microsatellite markers (ref. 17; BAT25, BAT26, NR21, NR22, and NR24) were used to evaluate MSI status. PCR products were analyzed in a 3130 ABI GeneScan. Those displaying DNA instability at only one of the markers (including the dinucleotides) were labeled MSI-L; those displaying instability at two or more markers were labeled MSI-H; and those displaying no instability at any of the five markers tested were labeled MSS. Due to unclear characteristics of MSI-L, we combined MSS and MSI-L into one group (non – MSI-H).

Methylation-specific PCR. The presence or absence of *hMLH1* (A and C promoter regions), *p16*, *APC*, and *APC2* methylation in cancers was determined by comparing the signals from tumor-derived tissues with those from normal, noncancerous tissues, as previously described (3, 4, 15, 16, 18). DNA was modified using the EZ DNA modification Gold kit as recommended by the manufacturer (Zymo Research). Methylation-specific PCR was used to distinguish unmethylated from methylated DNA, based on sequence alterations produced by bisulfite treatment of DNA, to convert unmethylated cytosines to uracil. These changes were identified by subsequent PCR using primers specific to the methylated (unchanged) or unmethylated (changed) DNA. PCR reactions were done using the primer pairs described below in the following reaction mix: 1× PCR buffer, deoxynucleotide triphosphates (each at 200 μmol/L), primers (0.1 μmol/L each per reaction), 0.5 unit of HotStart Taq DNA polymerase (Qiagen), and 50 ng of bisulfite-modified DNA in a final volume of 20 μL. Amplification was carried out in an Applied Biosystem temperature cycler. PCR cycling conditions were as follows: 95°C for 15 min, 35× [30 s at 95°C, 30 s (see Table 1) then 30 s at 72°C], followed by a final 4-min extension at 72 °C. The primer sequences and the annealing temperatures are listed in Table 1. Controls for methylated and unmethylated DNA were DNA from the SW48 colon cancer cell line and normal lymphocyte, respectively. Universally methylated DNA from Chemicon was also used as a positive control for some markers (CpG Genome, Chemicon). Each PCR reaction product (10 μL) was directly loaded into a 2% agarose gel, which was later stained with ethidium bromide to allow DNA visualization under UV illumination. The presence of a band in unmethylated tumor and matched normal with the absence of a methylation band in the tumor was defined as unmethylated. However, when a methylated band was present for tumor and absent for the normal, we defined the sample as methylated (or semi-methylated if both unmethylated or methylated bands were present in the tumor).

Histopathologic analysis. Independent pathologists who were unaware of the MSI status of any of the samples evaluated specific histopathologic characteristics. Grading of tumors was achieved by staining with H&E. Tumors were classified as proximal (proximal to the splenic flexure) or distal. The tumor-node-metastasis system of the International Union against cancer was used for tumor staging. Mucin production was evaluated using the modified criteria of Wiggers et al. and reported as absent (no extracellular mucin production), focal (when extracellular mucin production was present in <50% of the cells), or predominant (when the area of extracellular mucin production was present in ≥50% of the cells).

Immunohistochemistry. Tissue obtained from paraffin-embedded blocks was used for the immunohistochemistry experiments. Sections (5 μm) were mounted on charged glass slides, deparaffinized with xylene for 2 × 10 min, and rehydrated using a graded ethanol series. Antigen retrieval was done by placing the samples in a microwave oven for 12 min, with occasional interruption to avoid tissue degradation by excessive heat. The slides were then treated with hydrogen peroxide, followed by incubation with the primary and secondary antibodies, a streptavidin-biotin complex, an amplification reagent, streptavidin-peroxidase, and substrate-chromogen solution using the EnVision Systems according to the manufacturer's protocol (DAKO). The samples were then counterstained with hematoxylin, rinsed with ethanol, dried, and visualized under a light microscope. Tissue samples to which no primary antibody had been added were used as negative controls. All immunohistochemistry reagents were purchased from DAKO and the antibodies (hMLH1 clone G168-15, 1:100 dilution and hMSH2 clone 556349, 1:500 dilution) were purchased from Pharmingen. The slides were read by pathologists unaware of the MSI status. Positive staining was defined as >90% of cells staining for either MLH1 or MSH2, and negative staining was defined as no staining or <10% of the cells stained.

BRAF and KRAS mutation analysis. Samples were analyzed for the presence of a point mutation that frequently takes place in the oncogene BRAF, leading to the change of valine to glutamic acid at position 600 of the BRAF protein. DNA from the analyzed samples was used as a template in PCR reactions using two *BRAF* primers encompassing *BRAF* exon 15, one of which was biotinylated. The amplified fragments were then sequenced using a *BRAF* sequencing primer

Table 1. Methylation-specific primers and PCR conditions (number of cycle 35)

Genes	M/U	Primer sequences (5′→3′)	Annealing temperature (°C)	Amplicon size (bp)	Ref.
APC	U	F GTGTTTTATTGTGGAGTGTGGGTT R CCAATCAACAAACTCCCAACAA	61	108	(25)
	M	F TATTGCGGAGTGCGGGTC R TCGACGAACTCCCGACGA	61	98	
APC2	U	F TGGTAGTGTTGTTTGTTTAGGTTTGGATTG R ACCAAAAATCCCAACCCAAAATAACCTCAAAACA	56	102	(15)
	M	F GTCGTTTGTTTAGGTTCGGATC R GACCCGAAATAACCTCGAAACG	56	98	
MLH1 A-region	U	F TTTTGATGTAGATGTTTTATTAGGGTTGT R ACCACCTCATCATAACTACCCACA	61	124	(26)
	M	F ACGTAGACGTTTTATTAGGGTCGC R CCTCATCGTAACTACCCGCG	61	112	
MLH1 C-region	U	F GAAGAGTGGATAGTGATTTTTAATGT R ACTCTATAAATTACTAAATCTCTTCA	61	102	(28)
	M	F AGCGGATAGCGATTTTTAACGC/ R ACTCTATAAATTACTAAATCTCTTCG	61	98	
P16	U	F TTATTAGAGGGTGGGGTGGATTGT R CAACCCCAAACCACAACCATAA	61	151	(27)
	M	F TTATTAGAGGGTGGGGCGGATCGC R ACCCCGAACCGCGACCGTAA	61	150	

in a Biotage Pyrosequencer. The data were analyzed using Biotage SNP software. Tumor samples were also analyzed for KRAS codon 12 and 13 mutations. Both PCR and sequencing primers were purchased from Biotage. The generated pyrograms were also analyzed with the Biotage SNP program (Biotage).

Statistical analysis. The MSI phenotype was grouped into two levels: non–MSI-H (MSS + MSI-L) and MSI-H (instability at two or more markers). Age of patients was categorized into two groups: ≤60 and >60 y. The following categorical variables were used: stage of the cancer; gender; tumor location; mucin production; differentiation; methylation of *P16*, *hMLH1*, *hMSH2*, *APC*, and *APC2*; and immunohistochemistry results. For statistical analysis, mucin production was categorized as 0 (no production, <50%) or 1 (mucin producer, ≥ 50%). The distributions of categorical variables were shown by frequency in Tables 2 and 3. All analyses were done by the SPSS program 15.0.

Results

Clinicopathologic characteristics of the colorectal cancer tumors. Here we analyzed 222 colorectal tumors from AA patients and the clinicopathologic data are summarized in Table 2. Briefly, the gender distribution in our sample was 128 females (57.7%) and 94 males (42.3%). A majority (64.9%) of the patients were older than 60 years, whereas only 35.1% were younger than that age. There was no major predominance in tumor location, with the tumors more distributed to the proximal (47.3%) than distal (42.8%) colon. Stage-wise, there was a clear predominance of stages II and III, with ~60% of tumors being located. A similar pattern was seen with differentiation, with 71.6% of tumors being moderately differentiated. Most of the tumors (86.9%) in this study were non–mucin producing.

MSI status in the tumors. Of 222 colorectal tumors, 44 (19.8%) displayed instability in at least two of the five microsatellite markers used in this study and were therefore classified as MSI-H. Tumor samples showing stability at all five markers (138 MSS) or instability at no more than one marker (40 MSI-L) were grouped as non–MSI-H and represented 80.2% (178 cases) of all tumors in this study (Table 3). A comparison of the demographic and clinicopathologic data between the MSI-H and non–MSI-H groups did not reveal any statistically significant differences of any parameter in the two groups except for tumor differentiation. Most MSI tumors (71%) were moderately differentiated, whereas only two (5%) were well differentiated (Table 4).

Promoter methylation of APC, APC2, p16, and MLH1. The promoter regions of four different genes with different levels of relevance to colon carcinogenesis were analyzed by methylation-specific PCR to reveal their degree of methylation and epigenetic silencing. A p16 methylation study revealed that in 112 samples, 74.2% were not methylated, whereas only 25.8%

Table 2. Demographic and clinical characteristics of 222 colon cancer AA subjects

	Frequency (%)
Sex	
Female	128 (57.7)
Male	94 (42.3)
Age group	
≤60	78 (35.1)
>60	144 (64.9)
Total	222 (100.0)
Tumor location	
Left colon	95 (42.8)
Right colon	105 (47.3)
Unknown or multiple	22 (9.9)
Tumor stage	
0	2 (0.9)
I	19 (8.6)
II	65 (29.3)
III	68 (30.6)
IV	17 (7.7)
Unknown	51 (23.0)
Tumor differentiation	
Poor	21 (9.5)
Moderate	159 (71.6)
Well	16 (7.2)
Unknown	26 (11.7)
Mucin	
Non–mucin producing	193 (86.9)
Mucin producing	29 (13.1)

Table 3. The molecular signature of 222 colon cancer AA cases

	Frequency (%)
MSI	
Non – MSI-H	178 (80.2)
MSI-H	44 (19.8)
P16 methylation	
Unmethylated	81 (74.2)
Methylated	31 (25.8)
hMLH1 methylation	
Unmethylated	50 (33.3)
Methylated	101 (66.7)
APC methylation	
Unmethylated	32 (47.1)
Methylated	39 (52.9)
APC2 methylation	
Unmethylated	6 (10.1)
Methylated	50 (89.9)
hMLH1 IHC	
Negative	17 (12.8)
Positive	116 (87.2)
MSH-2 IHC	
Negative	13 (9.4)
Positive	125 (90.6)
BRAF mutation	
Wild	91 (92.9)
Mutated	7 (7.1)
KRAS mutation	
Wild	65 (69.1)
Mutated	29 (30.9)

Abbreviation: IHC, immunohistochemistry.

displayed methylation. Of 71 samples analyzed for *APC* gene methylation, 47.1% were unmethylated, whereas 52.9% were methylated. The situation was different with the *APC2* gene, where in 56 analyzed samples, 89.9% displayed methylation and only 10.1% were unmethylated. A quite similar methylation profile was observed with the DNA mismatch repair gene *MLH1*. In 151 samples, 66.7% were methylated and 33.3% were not using primers specific for the A region (Table 5).

DNA from a portion of available tumors that were either positively or negatively stained with MLH1 antibody by immunohistochemistry were used for MLH1 promoter methylation of the C-region (proximal region relative to the transcription start of the *MLH1* gene) experiments (19, 20). The goal of this experiment was to find whether or not the methylation status of this region might reveal the relevant specific promoter region for MLH1 expression. Fifty-eight percent of these tumors were methylated at the C-region (57.7%). Sixty percent of these methylated tumors were also MSI-H, whereas 40% were non – MSI-H. Seventy-three percent of the *MLH1* C-region methylated tumors were negatively stained for MLH1 in immunohistochemistry experiments, whereas only 26.7% were positively stained. Within each of the two groups of tumors, the association of the methylated *MLH1* C-region with MLH1 expression was 84.6% and 30.7% in the negatively and positively stained samples, respectively. MLH1, p16, APC, and APC2 promoter methylation profiles did not show any statistically significant differences in the MSI-H versus non – MSI-H group. *APC* showed a higher methylation level in MSI-H (70%) versus the non – MSI-H group (52%; Table 4) but the difference was not significant.

Effect of MLH1 and MSH2 expression on the MSI phenotype. Two DNA mismatch repair proteins, MLH1 and MSH2, were targeted in this study for their high relevance in the generation of the MSI phenotype and as a result of promoter methylation that blocks the expression of the genes in question. Of 133 samples, 87.2% stained positively for the MLH1 antibody, whereas 12.8% were negative. A similar profile was

Table 4. Relationship between demographic, clinical characteristics, and MSI status

	Non – MSI-H	MSI-H	*P*
Sex			
Female	101 (57)	27 (61)	0.58
Male	77 (43)	17 (39)	
Tumor stage			
0	2 (2)	0	0.25
I	18 (14)	1 (3)	
II	47 (36)	18 (46)	
III	53 (40)	15 (38)	
IV	12 (8)	5 (13)	
Tumor location			
Left colon	78 (48)	17 (44)	0.59
Right colon	83 (52)	22 (56)	
Mucin			
Non – mucin producing	157 (88)	36 (82)	0.26
Mucin producing	21 (12)	8 (18)	
Tumor differentiation			
Poor	11 (7)	10 (24)	0.007
Moderate	129 (84)	30 (71)	
Well	14 (9)	2 (5)	
Age group (y)			
≤60	61 (34)	17 (38)	0.59
>60	117 (66)	27 (61)	

Table 5. Relationship between molecular characteristics and MSI status

	Non – MSI-H	MSI-H	*P*
P16 methylation			
Unmethylated	68 (76)	21 (68)	0.35
Methylated	21 (24)	10 (32)	
hMLH1 methylation			
Unmethylated	44 (37)	6 (20)	0.07
Methylated	76 (63)	25 (80)	
APC methylation			
Unmethylated	29 (48)	3 (30)	0.30
Methylated	32 (52)	7 (70)	
APC2 methylation			
Unmethylated	4 (9)	2 (20)	0.30
Methylated	42 (91)	8 (80)	
hMLH1 IHC			
Negative	0	17 (46)	0.0001
Positive	96 (100)	20 (54)	
MSH-2 IHC			
Negative	0	13 (33)	0.0001
Positive	99 (100)	26 (67)	
PMS-2 IHC			
Negative	3 (9)	0	0.92
Positive	32 (91)	1 (100)	
BRAF mutation			
Wild	68 (100)	23 (77)	0.0001
Mutated	0	7 (23)	
KRAS mutation			
Wild	39 (64)	26 (79)	0.14
Mutated	22 (36)	7 (21)	

also obtained with MSH2, where of 138 samples, 90.6% stained positively for the MSH2 antibody and 9.4% were negative (Table 3). MSI-H and non–MSI-H groups displayed statistically different levels of staining for both MLH1 and MSH2, with the MSI-H group showing more negative stains. Forty-six percent of MSI-H cases were MLH1 negative, versus 0% for non–MSI-H cases. For MSH2, these values were 33% and 0%, respectively ($P = 0.0001$; Table 5).

KRAS and BRAF mutually exclusive in MSI tumors. Known mutations in the *KRAS* codons 12 and 13 and *BRAF* V600E oncogene were analyzed in this study. Of 98 analyzed tumors, 7 displayed the *BRAF* V600E point mutation, whereas of 94 samples analyzed for the *KRAS* mutation, 65 were wild-type and 29 samples displayed codon 12 and 13 mutations (Table 3). All found *BRAF* mutations were within the MSI-H group, whereas 21% of MSI-H tumors had *KRAS* mutations (Table 5). It is noteworthy that 36% of the *KRAS* mutated samples were non–MSI-H tumors.

APC, APC2, p16, and MLH1 methylation and tumor location. The methylation silencing of p16 was correlated with the proximal tumors but it was not statistically significant (Table 6). MLH1, APC, and APC2 methylation was equally distributed with regard to tumor location.

Relationship between BRAF or KRAS mutation and APC, APC2, p16, and MLH1 methylation. The BRAF and KRAS mutations analyzed were also correlated with gene silencing of *p16*, *MLH1*, *APC*, and *APC2* methylation (Tables 7 and 8). Unmethylated *MLH1* was significantly correlated with *BRAF* mutation, which may indicate that *MLH1* methylation may arise from a pathway other than *BRAF* signaling.

Discussion

In the current study, we investigated genetic and epigenetic pathways and markers for colorectal cancer in AAs, with the goal of finding some preferential colorectal carcinogenic pathways specific to this population. Future therapies could then be devised to target such pathways. Our previous reports (3, 18) have highlighted the high level of MSI found in AA colorectal cancers, but a larger sample size of colorectal cancer patients was needed to validate these findings. Here, we analyzed 222 colorectal cancer samples from AAs, and the MSI level was 19.8%, still higher than the U.S. general population (10-12%; ref. 21).

Table 6. P16, APC, APC2, and MLH1 methylation and tumor location

	Left colon	Right colon	*P*
p16			
Unmethylated	34 (42)	48 (58)	0.33
Methylated	8 (31)	18 (69)	
hMLH1			
Unmethylated	19 (39)	30 (61)	0.15
Methylated	44 (48)	47 (52)	
APC			
Unmethylated	18 (56)	14 (44)	0.53
Methylated	19 (49)	20 (51)	
APC2			
Unmethylated	2 (33)	4 (67)	0.39
Methylated	26 (52)	24 (48)	

Table 7. Relationship between KRAS mutation and gene methylation

	KRAS		*P*
	Wild	Mutated	
P16			
Methylated	28 (80)	10 (63)	0.18
Unmethylated	7 (20)	6 (37)	
hMLH1			
Methylated	14 (26)	9 (35)	0.42
Unmethylated	40 (74)	17 (65)	
APC			
Methylated	12 (43)	5 (36)	0.66
Unmethylated	16 (57)	9 (64)	
APC2			
Methylated	4 (21)	0 (0)	0.14
Unmethylated	15 (79)	11 (100)	

Whereas the difference in MSI level in AA colorectal cancer has been confirmed using a larger sample size, the MSI level we found in the present study is lower than those we found in previous reports, which were around 40% (3, 18). No association was found between the MSI phenotype and any of the demographic (age and gender) and clinicopathologic features (sex, age, tumor location, stage, and mucin production) except for a higher rate of poorly differentiated tumors among MSI-H. Indeed, in hereditary nonpolyposis colon cancer patients, colorectal cancer occurs as a result of mutated DNA mismatch repair genes independently of other parameters. The mutated or silenced DNA genes would then trigger the carcinogenic process by creating an environment that is prone to a high level of mutation. Our findings point to a higher importance of DNA mismatch repair genes in AA colorectal tumorigenesis. In addition, our data indicate the importance of *BRAF* and *KRAS* mutations in the MSI tumors (21, 22). *MSI*, *BRAF*, and *KRAS* mutations, as well as promoter methylation, were unevenly distributed within the tumors. All *P* values pointed to non-statistically significant differences, except for BRAF mutation in MSI-H, and to a lack of expression of MLH1/MSH2 and the MSI phenotype (<0.0001). These data suggest that colon cancer can be divided into distinct groups consistent with other studies in which integrated genetic and epigenetic analysis showed that colon cancers correspond to three molecularly distinct

Table 8. Relationship between *BRAF* mutation and gene methylation

	BRAF		*P*
	Wild	Mutated	
P16			
Methylated	42 (75)	4 (67)	0.66
Unmethylated	14 (25)	2 (33)	
hMLH1			
Methylated	26 (37)	1 (14)	0.23
Unmethylated	45 (63)	6 (86)	
APC			
Methylated	22 (46)	0 (0)	0.55
Unmethylated	26 (54)	1 (100)	
APC2			
Methylated	4 (10)	0 (0)	0.90
Unmethylated	35 (90)	1 (100)	

subclasses of disease (21) and in breast cancers, where hormone status and HER2 amplification define distinct groups (17).

It is well established that MSI results from nonfunctional or silenced DNA mismatch repair enzymes. Indeed, the immunohistochemistry data have shown that there is a strong and statistically significant correlation between the MSI-H status and negative stains for either MLH1 or MSH2. Mutations affecting these two genes or lack of their expression has been associated with MSI-H tumors. For some MSI-H tumors, both MLH1 and MSH2 were expressed. The genes for these two proteins might need to be sequenced to find any mutations that might have made these genes or their products nonfunctional, leading to the MSI-H phenotype. Other DNA mismatch repair genes, *PMS2* and *MSH6*, have also been associated with MSI status, although to a much lower degree than MLH1 and MSH2. The lack of MLH1 and MSH2 expression might be due to the methylation of these genes as well as to some other epigenetic processes involving chromatin modification. Our results with MLH1 methylation displayed a mild difference between MSI-H (80%) and non–MSI-H (63%) groups. Both groups displayed methylation above 60%. This high level of methylation in the non–MSI-H group does not correlate well with the positive stains obtained in this group with the MLH1 antibody. This discrepancy might be explained by the fact that there are many factors that do intervene in gene expression besides promoter methylation. Indeed, for the *MLH1* gene it has been reported that there are other CpG areas in the gene: the one we analyzed above in most of the samples in this study (A-region) and a second one (C-region) of which the methylation seems to play a major role in the regulation of MHL1 expression (19, 20). Our analysis of the MLH1 C-region methylation status has indeed revealed a stronger link between methylation, MSI phenotype, and MLH1 expression. More than 84% of the negatively stained tumors in MLH1 immunohistochemistry experiments were indeed methylated at this area of the promoter, whereas only 30% of the positively stained tissue displayed such methylation. In addition, 60% of MSI-H samples were methylated at this region, versus 40% for non–MSI-H tumors. As such, in our sample of AA patients, MLH1 C-region proves to be more specific in the mismatch repair gene expression than the A-region of MLH1 promoter region. In addition, it should be noted that the specificity of the methylation depends on the use of the combination of more specific primers that covers the *MLH1* promoter regions (19). In addition, there was no direct concordance between MLH1 methylation and MLH1 immunohistochemistry data in tissue containing methylation in the C-region. It is noteworthy that there are other epigenetic processes that might target this gene level of expression, processes that target the chromatin structure making it more relaxed or tighter. These events involve the chemical structure of histones (acetylation, methylation, phosphorylation, and sumoylation) and affect their affinity to the DNA double helix. In addition, the effect of miRNA on suppression of genes, such as *APC*, without mutation and/or methylation has been recently recognized (23). As such, the level of expression of a given gene will be the result of all these processes together. We have shown that in AAs, there are global H3 and H4 acetylation and an HDAC2 expression profile that is important in the progression of normal tissue to a tumor phenotype (24).

The methylation profile for the *APC* and *APC2* genes was not different between the MSI-H and non–MSI-H groups. A higher methylation level was observed for the *APC2* gene in comparison with *APC*. Both genes encode adenomatous polyposis coli (FPC) tumor suppressor proteins. It is well known that *APC* mutations are the driving force in FPC colorectal cancer. Therefore, it is not surprising to find this high level of loss of expression in the analyzed sample. The high methylation levels of these two genes combined with the high level of MSI might play a critical role in the high incidence of colorectal cancer in AAs because both events occur early in colorectal carcinogenesis and also would lead to fast-developing, highly invasive tumors, which characterize AA colorectal cancer.

The *p16* gene promoter was the least methylated of the genes analyzed in this study, confirming our findings from our previous reports. This gene is involved in cell cycle regulation and negatively regulates cyclin-dependent kinase 4 and is directly involved in cell proliferation. One third of all analyzed tumors displayed methylation of this gene, which makes it relevant to the colorectal cancer process in AAs but not at the level of the *APC* genes, which displayed much higher methylation rates up to 90%. Unlike MLH1, APC, and APC2 for which the methylation was equally distributed all over the colon, p16 methylation was twice as frequently located in the right colon (67%) as in the left side (Table 6). As such, the predominant methylation in proximal tumors can only be attributed to this marker but not to the other ones.

Besides tumor suppressor genes, oncogenes also play a major role in the carcinogenic process. Our results show that at least one quarter of the MSI-H tumors have the *BRAF* 600 mutation, whereas none of the non–MSI-H tumors have it. This result correlates with findings that many MSI tumors develop through the *BRAF* pathway, although no mechanistic relationship was established. However, our analysis of 94 samples has shown that unlike the *BRAF* mutation, the *KRAS* codon 12 and 13 mutation is distributed equally in all tumors regardless of their MSI status. This finding is very important because none of the MSI-H tumors carried *BRAF* and *KRAS* mutations simultaneously. As such, the mutations that hit these two genes are reciprocally exclusive and may be linked to different mutational processes targeting the same signal transduction pathway requiring an activating mutation at either one of the two markers.

This study on AA colorectal cancers has revealed some race/ethnicity-specific information about colorectal cancer such as the high MSI level. Like colorectal cancer in other groups, the same set of genes seem to be recruited in the process of carcinogenesis including TSG (*p16*, *APC*, and *APC2*) as well as oncogenes (*BRAF* and *KRAS*), but with a distinct pattern. With the advent of high-throughput technologies and wide genomic assay including genome SNP analysis, expression microarray, and array comparative genomic hybridization, it is possible to carry out global genome analysis from different ethnic groups. This information could help us understand colorectal cancer carcinogenesis within different populations and help us more efficiently target the disease according to the markers that play a major role in the disease process of colorectal cancer. Further studies will be needed to analyze the epidemiology and clinical courses of these subclasses of colon cancers, including the future prognostic use of our findings.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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