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Concordant CpG Island Methylation in Hyperplastic Polyposis

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The CpG island methylator phenotype (CIMP) is a newly described mechanism for carcinogenesis in colorectal carcinomas and adenomas characterized by methylation of multiple CpG islands. The causes of CIMP are unknown. We studied CIMP in hyperplastic polyps (HPs), with emphasis on patients with multiple HPs (5 to 10 HPs), large HPs (one HP >1 cm) or hyperplastic polyposis (>20 HPs). Methylation of *p16*, MINT1, MINT2, MINT31, and *hMLH1* was analyzed by methylation-specific polymerase chain reaction in 102 HPs, 8 serrated adenomas, 19 tubular adenomas, and 9 adenocarcinomas from 17 patients, with multiple/large HPs or hyperplastic polyposis and in 16 sporadic HPs from 14 additional patients. Sporadic HPs were CIMP-negative (not methylated at any locus), but 43% of HPs from multiple/large HPs, or hyperplastic polyposis were CIMP-high (two or more methylated loci, $P = 0.00001$). Methylation among the four loci was correlated within HPs (odds ratio, 3.41; $P = 0.002$), and the methylation status of HPs within the same patient was also correlated (odds ratio, 5.92; $P = 0.0001$). CIMP-high HPs were present primarily in patients with a predominance of HPs in the right colon and/or serrated adenomas ($P = 0.0009$) and were associated with the absence of K-ras proto-oncogene mutations (odds ratio, 5.08; $P = 0.03$). Our findings of concordant CpG island methylation of HPs in multiple/large HPs or hyperplastic polyposis supports the concept that some patients have a hypermethylator phenotype characterized by methylation of multiple HPs and other colorectal lesions. The hypermethylator phenotype is related to patient-specific factors, such as carcinogenic exposure or genetic predisposition. *(Am J Pathol 2002, 160:529–536)*

Colorectal cancer is the second most common cause of cancer deaths in the United States. Most colorectal cancers develop from adenomatous polyps, and morphological and genetic progression in an adenoma-adenocarcinoma sequence and in hereditary colorectal cancer syndromes are well described.[1–5]

CpG islands are 0.5- to 2-kb regions rich in cytosine-guanine dinucleotides and are present in the 5′ region of approximately half of all human genes.[6] Methylation of cytosine within CpG islands is associated with loss of gene expression and is observed in physiological conditions, such as X chromosome inactivation[7] and aging,[8] and in neoplasia.[9] Transcriptional repression by methylation in colorectal cancers inactivates the *p16* cell-cycle regulator,[10] the estrogen receptor growth suppressor,[8] the *THBS1* angiogenesis inhibitor,[11] the *TIMP3* metastasis suppressor,[12] the *O^6-methylguanine DNA methyltransferase* DNA repair gene,[13] and the *hMLH1* nucleotide mismatch repair gene.[14] The recently discovered CpG island methylator phenotype (CIMP) is a novel pathway characterized by methylation of multiple CpG islands in colorectal carcinomas and adenomas.[15–17] CIMP-high adenomas and carcinomas have a distinct genetic profile with frequent mutations of the *K-ras* gene, but lack of *p53* mutations.[16] CIMP status is not correlated among multiple adenomas from the same patient.[17] The mechanism of methylation of multiple CpG islands is postulated to be either aberrant *de novo* methylation because of mutation in a DNA-methyltransferase or loss of protection against *de novo* methylation through the loss of a *trans*-activating factor.[18–20]

Hyperplastic polyps (HPs) are usually present in the left colon, small in size, and considered to be benign in nature. However, patients with hyperplastic polyposis, characterized by the presence of numerous HPs and/or large HPs, have increased risk of colorectal cancer.[21–28] A HP-serrated adenoma-carcinoma sequence is proposed as an alternative pathway to the adenoma-carcinoma sequence.[28–31]

We investigated the possibility that CpG island methylation is a major molecular defect in patients with multiple/large HPs or hyperplastic polyposis, and report a high degree of patient-specific concordant methylation in HPs and other lesions, representing a hypermethylator phenotype.

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Materials and Methods

Characteristics of Patients and Specimens

The patients and specimens have been described in detail previously.[28] The patients were classified into three groups based on the number and size of HPs: multiple HPs (patients with 5 to 10 HPs), hyperplastic polyposis (patients with >20 HPs), and large HPs (patients with HP >1 cm), as described previously.[28] Predominance of HPs in the right colon and predominance of HPs in the left colorectum were defined by the location of the majority of HPs in the right colon or in the left colon and rectum, respectively. We initially studied 129 HPs from 23 patients. All histological sections had limited quantities of DNA extracted from tissue in paraffin-embedded specimens. Twenty-seven HPs, eight tubular adenomas, and three carcinomas from three patients with familial hyperplastic polyposis and three patients with hyperplastic polyposis from the previous study were excluded because of insufficient DNA for further analysis. Thus, we studied 102 HPs, 8 serrated adenomas, 19 tubular adenomas, and 9 carcinomas from 17 patients with hyperplastic polyposis. Histologically normal mucosa was available from 13 patients.

Sixteen sporadic HPs from 14 patients undergoing resection of colorectal cancer at the MD Anderson Cancer Center, Houston, TX, were also analyzed. All patients had given informed consent for the collection of specimens according to institutional guidelines.

Bisulfite Treatment of DNA and Methylation-Specific Polymerase Chain Reaction

The methylation status of *p16*, MINT1, MINT2, MINT31, and *hMLH1* was determined by bisulfite treatment of DNA followed by methylation-specific polymerase chain reaction, as described with modification.[32] These loci were chosen based on a previous study that showed that they offered excellent discrimination for CIMP and that they were unmethylated (<10% methylation) in normal tissues.[15]

In brief, 2 μg of microdissected genomic DNA was denatured with 2 mol/L NaOH at 37°C for 10 minutes, followed by incubation with 3 mol/L sodium bisulfite (pH 5.0) at 50°C for 16 hours in the dark. DNA was then purified using the DNA Cleanup Kit (Promega, Madison, WI) as recommended by the manufacturer, incubated with 3 mol/L of NaOH at room temperature for 5 minutes, precipitated with 10 mol/L of ammonium acetate and 100% ethanol, washed with 70% ethanol, and finally resuspended in 20 μl of distilled water.

The primers and polymerase chain reaction (PCR) conditions for *p16* were the same as reported by Herman and colleagues.[32] The primers for MINT1 were 5′-AATTTTTTTATATATATTTTCGAAGC-3′ and 5′-AAAAAC-CTCAACCCCGCG-3′ for methylated alleles; and 5′-AAT-TTTTTTATATATATTTTTGAAGTGT-3′ and 5′-AACAAA-AAACCTCAACCCCACA-3′ for unmethylated alleles. The cycling conditions for MINT1 were 95°C for 10 minutes and 37 cycles of 95°C for 30 seconds and 55°C for 45 seconds. The primers for MINT2 were 5′-TTGTTAAAGTGTTGAGT-TCGTC-3′ and 5′-AATAACGACGATTCCGTACG-3′ for methylated alleles; and 5′-GATTTTGTTAAAGTGTTGAGTT-TGTT-3′ and 5′-CAAAATAATAACAACAATTCCATACA-3′ for unmethylated alleles. The cycling conditions for MINT2 were 95°C for 10 minutes and 40 cycles of 95°C for 30 seconds and 60°C for 45 seconds. The primers for MINT31 were 5′-TGTTGGGGAAGTGTTTTTCGGC-3′ and 5′-CGAAAACGAAACGCCGCG-3′ for methylated alleles; and 5′-TAGATGTTGGGGAAGTGTTTTTTGGT-3′ and 5′-TA-AATACCCAAAAACAAAACACCACA-3′ for unmethylated alleles. The cycling conditions for MINT31 were 95°C for 10 minutes and 38 cycles of 95°C for 30 seconds and 60°C for 45 seconds. The primers for *hMLH1* were 5′-GATAGCGATTTTTAACGC-3′ and 5′-TCTATAAATTACTA-AATCTCTTCG-3′ for methylated alleles; and 5′-AGAGTG-GATAGTGATTTTTAATGT-3′ and 5′-ACTCTATAAATT-ACTAAATCTCTTCA-3′ for unmethylated alleles. The cycling conditions for *hMLH1* were 95°C for 10 minutes and 40 cycles of 95°C for 30 seconds and 53°C for 45 seconds.

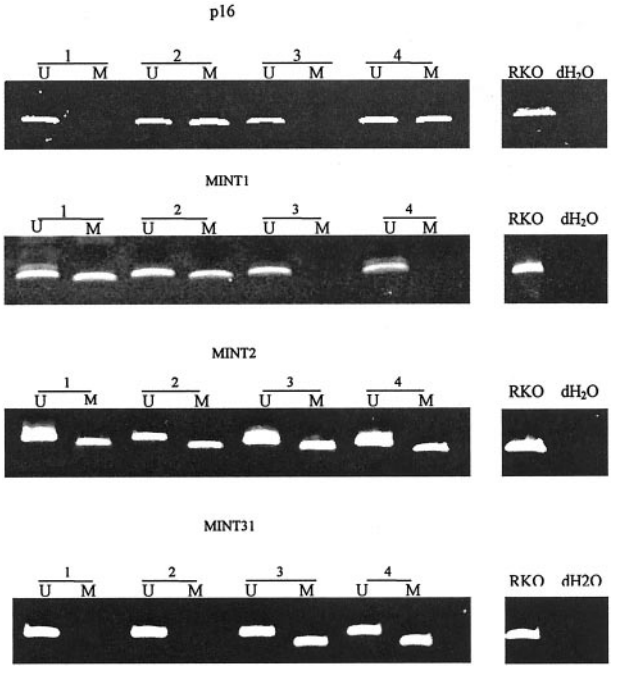

Figure 1. Methylation analysis of CpG islands in HPs. Methylation of *p16*, MINT1, MINT2, and MINT31 was evaluated by methylation-specific PCR using primers for methylated (M) and unmethylated (U) alleles of bisulfite-treated DNA. Loci examined and HP numbers are indicated above each gel. DNA from RKO, a colon cancer cell line, was used as a positive control, and dH_2O without DNA was used as negative control.

In brief, 2 μl of bisulfite-treated DNA was used as template for PCR reactions using primers specific for methylated and unmethylated alleles. RKO, a colon cancer cell line (American Type Culture Collection, Manassas, VA), was used as a positive control, and PCR reaction without DNA was used as the negative control in each batch of reaction. PCR products from methylated and unmethylated reactions were electrophoresed on 6% acrylamide gels and visualized by ethidium bromide staining (examples in Figure 1). For quantitation of methylated and unmethylated alleles, gel photographs were

digitized using a BioRad imager (Bio-Rad, Richmond, CA) and evaluated by densitometry using the manufacturer's software. The results were expressed as percentage of methylation by determining the density of the methylated band relative to the sum of the methylated and unmethylated bands. The loci used in this study are unmethylated (<10% methylated) in normal tissues. Therefore, any locus showing ≥10% methylation was considered positive.

CIMP Status of HPs, Serrated Adenomas (SAs), Adenomas, and Carcinomas

HPs, SAs, adenomas, and carcinomas were classified as CIMP-negative if none of the loci were methylated; CIMP-low, if one locus was methylated; and CIMP-high, if two or more loci were methylated.

K-ras Mutations, Loss of Heterozygosity of Chromosome 1p, and Microsatellite Instability-High (MSI-High)

K-ras mutations, loss of heterozygosity of chromosome 1p, and MSI in HPs and SAs from patients with hyperplastic polyposis were reported previously.[28] MSI-high was defined by presence of allelic shift in comparison with control DNA in at least 30% of evaluated markers.

Statistical Analysis

The primary statistical endpoint of this study was the determination of factors associated with CIMP status of HPs. Patients with more than one HP were represented multiple times in this data set. Each HP was represented by a methylation index (number of loci methylated/number of loci evaluated). To model correctly the within-polyp and between-polyp correlation as well as simultaneously partition out the effects of the various factors considered, marginal logistic regression models for correlated binary data were used to assess associations between CIMP status and the various polyp and patient characteristics. Estimates were obtained using the generalized estimating equation approach of Zeger and Liang.[33] An appropriate correlation structure was chosen to account for possible correlations both between HPs within patients and within HPs between observations from different loci. Both patient and polyp characteristics were tested for association with methylation status. The factor locus was also included in the model to account for locus-specific methylation rates. Relationships between HPs within patients and within HPs between loci were represented as odds ratios, in which an odds ratio of greater than one suggests positive correlation in CIMP status within patients and within polyps, respectively.

Results

There were 11 men and 6 women with multiple/large HPs, or hyperplastic polyposis. The mean age was 64 ± 12 years (range, 46 to 84 years). The demographic data and characteristics of each individual patient and the number of HPs, adenomas, and carcinomas in each individual are summarized in Table 1. The sporadic HPs were from 11 men and 3 women, with a mean age of 64 ± 11 years (range, 48 to 80 years). The mean size of the polyp in this group was 0.3 cm (range, 0.1 to 0.7 cm). There were 2 HPs from the right colon and 14 from the left colon.

Methylation of Sporadic HPs

None of the sporadic HPs were methylated at any of the four loci (Figure 2, A and B) and were classified as CIMP-negative.

Methylation of Nonlesional Mucosa, Polyps, and Cancers from Patients with Multiple/Large HPs, or Hyperplastic Polyposis

Nonlesional Mucosa

The apparently normal colorectal mucosa was analyzed for methylation in 16 samples from 13 patients (Figure 3). Only three patients had methylation (patients 4, 9, and 15) in the mucosa taken adjacent to a neoplasm: methylation of *p16* was present in mucosa adjacent to a tubular adenoma in patient 15, MINT1 was methylated in mucosa adjacent to a tubular adenoma in patient 9, and *p16* and MINT1 were methylated in mucosa adjacent to a carcinoma in patient 4. The methylation at the normal mucosae in these three patients was concordant with the adjacent adenoma or carcinoma. No methylation was present in seven samples from mucosa of the resection margins or in six additional samples of mucosa adjacent to a lesion. Thus, 6% (1 of 16) of normal mucosae were classified CIMP-high, 12% (2 of 16) were CIMP-low, and 81% (13 of 16) were CIMP-negative.

Hyperplastic Polyps

Forty-three percent (44 of 102) of HPs from these patients were classified as CIMP-high, 14% (14 of 102) were CIMP-low, and 43% (44 of 102) were CIMP-negative ($P = 0.00001$ *versus* sporadic HPs, and $P = 0.0045$ *versus* nonlesional mucosa; Figure 2, A and B; Figure 3).

The methylation statuses of different loci in the same HP were positively correlated (odds ratio, 3.41; $P = 0.002$; Figure 3), suggesting that some HPs from patients with multiple/large HPs, or hyperplastic polyposis, have CIMP-high.[15] In addition, CIMP statuses for different HPs within the same patient were positively correlated (odds ratio, 5.92; $P = 0.0001$), suggesting concordance of methylation in multiple HPs within individual patients.

The methylation status of HPs was tested for associations with patient characteristics, with age, the site and size of HPs, and with other genetic alterations in HPs

Table 1. Patient Demographics, Characteristics, and Histopathological Type of Lesions in Patients with Multiple/Large HPs, or Hyperplastic Polyposis

Patient	Age, yrs	Sex	Hyperplastic polyps, total/analyzed	Serrated adenomas, total/analyzed	Tubular adenomas, total/analyzed	Colorectal cancer
Patients with predominance of HPs in left colorectum without serrated adenomas						
1	54	F	4*/4	—	—	−
2	62	M	Numerous/5	—	—	+
3	65	F	>100/3	—	5/4	+
4	76	F	>50*/3	—	1/1	+§¶
5	67	M	Numerous/14	—	2/0	+
6	54	M	8/7	—	3/0	+
7	51	F	10/7	—	—	+
8	61	M	8/3	—	1/0	+
Patients with predominance of HPs in right colon without serrated adenomas						
9	48	F	36/15	—	9/2	+
10	82	M	6†/6	—	4/3	+
11	84	M	2*/2	—	2/2	+¶
Patients with predominance of HPs in left colorectum with serrated adenomas						
12	57	M	22/4	2/2‡	4/1	+
13	55	M	13*/3	1/1‡	3/0	−
14	76	M	7/5	3/1	8/0	+
Patients with predominance of HPs in right colon with serrated adenomas						
15	78	M	9/8	4/3	6/2	−
16	46	M	26/10	1/1	8/2	+
17	68	F	Multiple*/3	1/0	2/2	+§¶

*Also had a large hyperplastic polyp.
†Four large hyperplastic polyps.
‡Admixed hyperplastic adenomatous polyp.
§Multiple, mucinous colon cancers.
¶Right-sided colon cancer.

(Table 2). There was no association between methylation and age. Patients with the three phenotypic groups of multiple HPs, large HPs, and hyperplastic polyposis had overlapping CIMP status in HPs from the same patient ($P = 0.8$; Figure 2C). HPs from patients with large HPs were invariably methylated, but HPs from patients with hyperplastic polyposis and multiple HPs as defined in the previous study[28] were heterogeneous for methylation status. HPs were more commonly methylated in patients with SAs, predominance of HPs in right colon, or both SAs and predominance of HPs in right colon ($P = 0.0009$; Figure 3 and Table 2). Methylation was present in 22% (10 of 46) of HPs in patients with predominance of HPs in left colorectum without SAs (odds ratio, 1.00), 100% (23 of 23) of HPs from patients with predominance of HPs in right colon without SAs (odds ratio, 27.55; 95% confidence limit, 7.33 to 103.60), 83% (10 of 12) of HPs from patients with predominance of HPs in left colorectum with SAs (odds ratio, 9.55; 95% confidence limit, 3.72 to 24.53), and 76% (16 of 21) of HPs from patients with predominance of HPs in right colon with SAs (odds ratio, 4.60; 95% confidence limit, 1.54 to 13.68).

The HP characteristics that associated with CIMP status of HPs were absence of K-ras mutations, absence of loss of heterozygosity of chromosome 1p, and site of HP in the right colon (Figure 3 and Table 2). K-ras mutations were present in 0% (0 of 44) of CIMP-high HPs, 21% (3 of 14) of CIMP-low HPs, and 16% (7 of 44) of CIMP-negative HPs (odds ratio, 5.08; $P = 0.03$). Similarly, loss of heterozygosity of chromosome 1p was present in 2% (1 of 44) of CIMP-high HPs, 7% (1 of 14) of CIMP-low HPs, and 11% (5 of 44) of CIMP-negative HPs (not significant). HPs in the left colon and rectum were less methylated than HPs in the right colon, and larger HPs were more frequently methylated. The site and size of HPs were not independently associated with methylation status but were dependent on patient characteristics.

Serrated Adenomas

Most of the SAs were methylated: 75% (6 of 8) of SAs were CIMP-high, 12% (1 of 8) were CIMP-low, and 12% (1 of 8) were CIMP-negative (Figures 2B and 3).

Tubular Adenomas and Carcinomas

The CIMP status of adenomas and carcinomas was more heterogeneous (Figures 2B and 3). CIMP-high was present in 32% (6 of 19) of adenomas, CIMP-low in 32% (6 of 19), and CIMP-negative in 37% (7 of 19). CIMP-high was present in 44% (4 of 9) of carcinomas, CIMP-low in 11% (1 of 9), and CIMP-negative in 44% (4 of 9) (Figures 2B and 3). All three right-sided colonic carcinomas were CIMP-high (patients 4, 11, and 17) as contrasted with one of six left-sided colorectal carcinomas (patient 12, $P = 0.05$). K-ras mutation was present in 5% (1 of 19) adenomas and in none of nine carcinomas.

MSI-High and Methylation of hMLH1

All HPs from patients with multiple/large HPs or hyperplastic polyposis were MSI-negative, but MSI-high was present in one SA, four tubular adenomas, and two car-

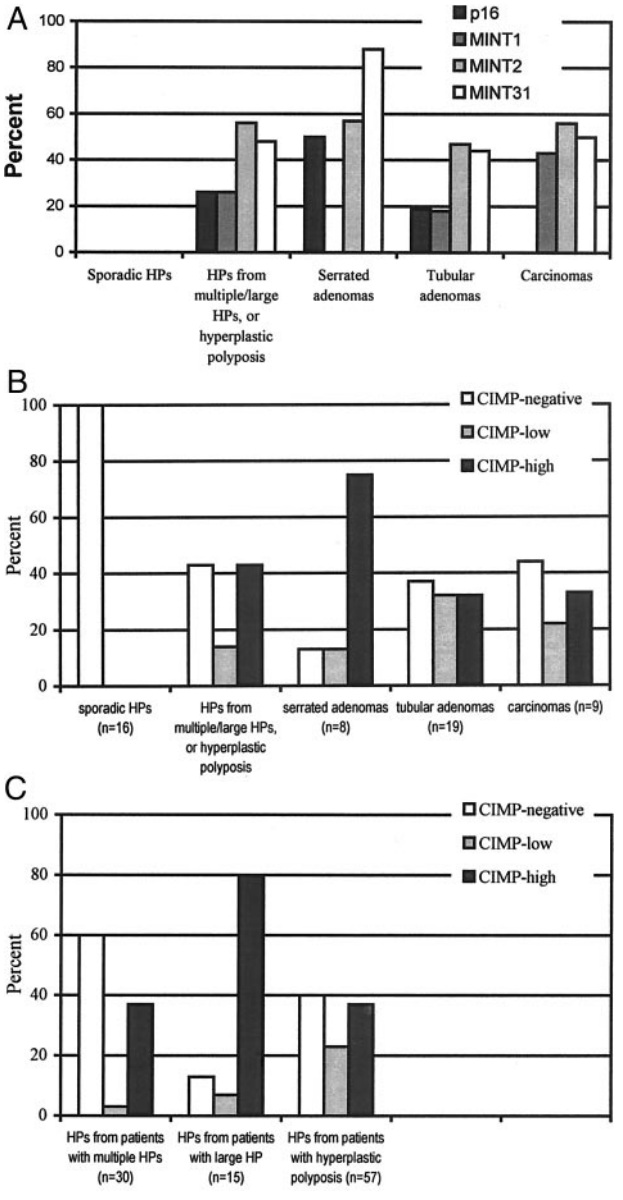

Figure 2. A: Prevalence of methylation at *p16*, MINT1, MINT2, and MINT31 in HPs, serrated adenomas, tubular adenomas, and carcinomas. **B:** CIMP status of sporadic HPs and of HPs, serrated adenomas, tubular adenomas, and carcinomas from patients with multiple HPs, large HPs, and hyperplastic polyposis. **C:** CIMP status of HPs from patients with multiple HPs, large HPs, and hyperplastic polyposis.

cinomas from four patients (patients 3, 4, 12, and 17; Figure 3). CIMP, MSI-high, and methylation of *hMLH1* status were compared in multiple lesions from the four patients with MSI-high SA, tubular adenomas, or colorectal carcinomas (Figure 4). Concordant methylation of *hMLH1*, CIMP-high, and MSI-high was present in an adenoma and carcinoma from one patient, and a carcinoma from another patient (patients 4 and 17), but methylation of *hMLH1* was not present in all other MSI-negative HPs, SAs, adenomas, or carcinomas. Methylation of *hMLH1* was not present in one CIMP-high, MSI-high SA from one patient (patient 12); and one CIMP-high and two CIMP-negative, MSI-high adenomas from another patient (patient 3).

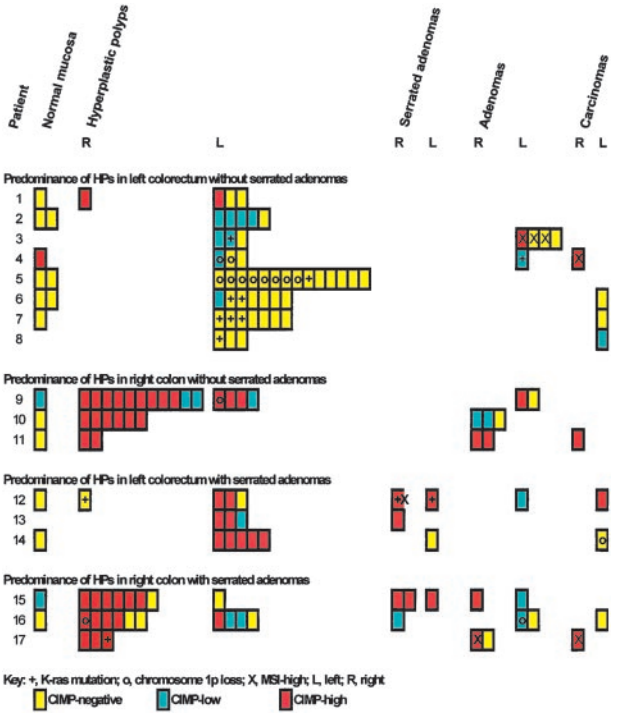

Figure 3. Methylation status of HPs, serrated adenomas, tubular adenomas, and carcinomas from patients with multiple HPs, large HPs, and hyperplastic polyposis.

Discussion

We studied CpG island methylation in HPs and other colorectal lesions from patients with multiple/large HPs, or hyperplastic polyposis, and in sporadic HPs from patients with colorectal carcinomas. Normal mucosa from the same patient and a group of sporadic HPs were used as controls. Methylation was present only in the mucosa dissected adjacent to a neoplasm that could have resulted from a field defect. No methylation was present in sporadic HPs nor the normal mucosa from the resection margins. CIMP-high was present in 43% of HPs from patients with multiple/large HPs, or hyperplastic polyposis, a percentage that is similar to the prevalence of CIMP-high in sporadic colorectal carcinomas and adenomas.[15–17]

A high degree of concordance was observed among HPs within the same patient with multiple/large HPs, or hyperplastic polyposis, in our study. In contrast, CIMP status was not correlated among sporadic adenomas within the same patient in another study.[17] These findings suggest that some of the patients with multiple/large HPs, or hyperplastic polyposis, may have a hypermethylator phenotype either because of genetic predisposition to develop methylated HPs and related lesions, or because of an environmental exposure that results in development of multiple methylated HPs. Effects of carcinogen exposures on CpG island methylation of estrogen receptor in lung carcinomas have previously been described.[34] This, together with the fact that most patients in the present study had no family history of colonic neoplasms, tends to favor the latter hypothesis.

Table 2. Patient and Polyp Characteristics in Relation to CIMP Status of Hyperplastic Polyps in Patients with Multiple/Large HPs or Hyperplastic Polyposis, Odds Ratio, and 95% Confidence Intervals (CI) from Generalized Estimating Equation Marginal Regression Models

Factors	CIMP-negative, % (fraction)	CIMP-low, % (fraction)	CIMP-high, % (fraction)	Odds ratio (CI)	Chi-square	*P* value
Patient characteristics						
With serrated adenoma						
Predominance of HPs in right colon	0 (0/23)	13 (3/23)	87 (20/23)	27.55 (7.3, 103.6)	16.52	0.009
Predominance of HPs in left colorectum	17 (2/12)	8 (1/12)	75 (9/12)	9.55 (3.7, 24.5)		
Without serrated adenoma						
Predominance of HPs in right colon	24 (5/21)	14 (3/21)	62 (13/21)	4.60 (1.5, 13.7)		
Predominance of HPs in left colorectum	78 (36/46)	17 (8/46)	4 (2/46)	1.00		
Age (continuous, years)	—	—	—	1.02 (0.98, 1.8)	1.52	0.22
Hyperplastic polyp characteristics						
Site						
Left	66 (38/58)	16 (9/58)	19 (11/58)	0.39 (0.1, 1.1)	2.63	0.10
Right	11 (5/44)	14 (6/44)	75 (33/44)	1.00		
Size (continuous, mean ± SD, mm)	3.0 ± 1.5	3.1 ± 1.6	5.1 ± 4.3	1.04 (0.9, 1.2)	0.70	0.40
K-ras mutation						
Absent	40 (37/92)	12 (11/92)	48 (44/92)	5.08 (1.5, 17.6)	4.72	0.03
Present	70 (7/10)	30 (3/10)	0	1.00		
1p loss						
Absent	41 (39/95)	14 (13/95)	45 (43/95)	3.24 (0.7, 15.7)	1.94	0.16
Present	71 (5/7)	14 (1/7)	14 (1/7)	1.00		

Methylation was more common in patients with predominance of HPs in the right colon and/or SAs. We have previously described differences in topographic expression of $p21^{Waf1/Cip1}$ cyclin-dependent kinase inhibitor and Ki-67 proliferation marker in right- and left-sided HPs from these patients.[28]

The genetic alterations in sporadic HPs differ from genetic alterations in HPs from patients with multiple HPs, large HPs, or hyperplastic polyposis. Sporadic HPs have frequent *K-ras* mutations and loss of chromosome 1p,[35–39] but lack CpG island methylation. In contrast, HPs from patients with multiple/large HPs, or hyperplastic polyposis, have infrequent *K-ras* mutations, and loss of chromosome 1p in a small percentage of HPs,[28] but frequent CpG island methylation. Furthermore, *K-ras* mutation or loss of chromosome 1p was predominantly present in HPs from patients with predominance of HPs in the left colorectum without serrated adenomas, a set of patients who lack CIMP-high HPs or carcinomas. These results suggest that either K-ras mutations and loss of chromosome 1p are a late event or two alternative sets of early genetic events occurring in HPs, one characterized by CpG island methylation and the other by K-*ras* mutations or loss of chromosome 1p. The latter is further corroborated by lack of K-ras mutations in adenomas and carcinomas in these patients.

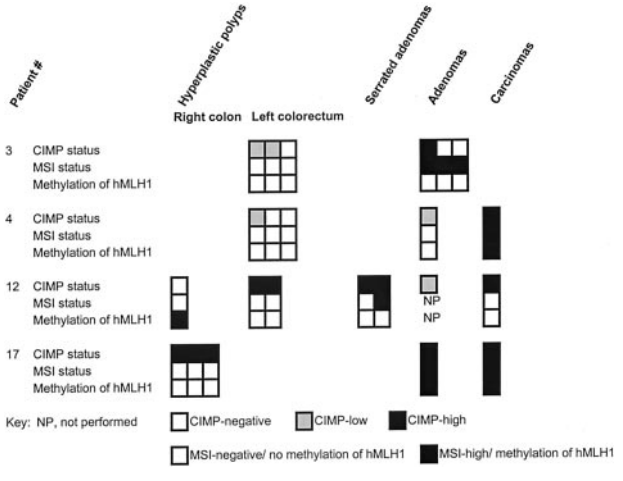

Figure 4. CIMP, MSI-high, and *hMLH1* methylation status of HPs, serrated adenomas, tubular adenomas, and carcinomas from patients with MSI-high lesions. Concordant methylation of *hMLH1*, CIMP-high, and MSI-high was present in an adenoma and carcinoma from one patient and a carcinoma from another patient, but methylation of *hMLH1* was not present in all other MSI-negative HPs, serrated adenomas, tubular adenomas, or carcinomas.

CIMP-high colorectal carcinomas and adenomas have frequent methylation of the *p16* gene and mutations of the *K-ras* gene, but lack *p53 gene* mutation.[16,40] HPs with CIMP-high from the present study had frequent methylation of the *p16* gene, but lacked *K-ras* mutations and p53 overexpression of the type seen with p53 gene mutation.[28] These data provide additional evidence that progression of colorectal carcinogenesis in patients with hyperplastic polyposis is distinct from sporadic CIMP-negative and CIMP-high carcinomas. There is evidence for two CIMP-positive pathways of colorectal carcinogenesis: one characterized by methylation of *O^6-methylguanine DNA methyltransferase* DNA repair gene is associated with MSI-low, and G to A *K-ras* and *p53* gene mutations;[13,41,42] and an another characterized by CIMP-high, paucity of *K-ras* gene mutation, and with or without MSI-high dependent on methylation status of *hMLH1*.[14–17,41]

MSI-high SA, tubular adenomas, or carcinomas were present in four patients in our study. Concordant methylation of *hMLH1*, CIMP-high, and MSI-high was present in one tubular adenoma and two carcinomas from two patients. Another patient had MSI-high, CIMP-high SA without methylation of *hMLH1*. All of these three patients had CIMP-high HPs without MSI. In contrast, one patient had MSI-high in three adenomas without CIMP-high in the majority of adenomas or HPs. There was no MSI-positive, CIMP-high HPs in our study. This data suggest that methylation of *hMLH1* marks a genetic and histological pro-

gression in CIMP-high lesions that develop MSI-high and undergo adenomatous or carcinomatous transformation in a few patients: CIMP-high tubular adenomas and carcinomas develop in patients with CIMP-high HPs because of methylation of *hMLH1*. This corroborates loss of expression of hMLH1 in MSI-high dysplastic and malignant foci in hyperplastic polyposis noted by Jass and colleagues.[31]

Thus, some patients with multiple/large HPs, or hyperplastic polyposis, have concordant methylation of multiple CpG islands in HPs. The patient factors that we determined to be important for the development of heavy methylation were the presence of SA(s) and the predominance of HPs in right colon. The role of *HPP1* gene methylation in the development of HPs corroborates our findings.[43] A CpG island methylation-independent pathway to hyperplastic polyposis also exists, as documented by four patients with loss of chromosome 1p or *K-ras* mutations in the majority of their HPs. Neoplastic progression because of microsatellite instability is also present in multiple/large HPs, or hyperplastic polyposis.[28–31]

A high degree of concordance of methylation is observed among HPs in multiple/large HPs or hyperplastic polyposis within the same patient, supporting the existence of a hypermethylator phenotype. Patients with a hypermethylator phenotype will be invaluable in studying and understanding the causes of aberrant methylation in cancer.

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