

# CpG Island Methylation in Aberrant Crypt Foci of the Colorectum

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**Aberrant crypt foci (ACF) are postulated to be the earliest precursor lesion in colorectal carcinogenesis, and CpG island methylation has been described as an important molecular pathway. We therefore studied methylation in ACF from patients with familial adenomatous polyposis (FAP) or sporadic colorectal cancer. We assessed methylation status of the *p16* tumor suppressor gene, MINT1 (methylated in tumor 1), MINT2, MINT31, *O*<sup>6</sup>-methylguanine-DNA methyltransferase gene, and *hMLH1* mismatch repair gene. We compared methylation to ACF histopathology, *K-ras* proto-oncogene mutation, loss of heterozygosity at chromosome 1p, and microsatellite instability. Methylation was present in 34% (21 of 61) of ACF, including both FAP and sporadic types, but was more frequent in sporadic ACF [53% (18 of 34) versus 11% (3 of 27), *P* = 0.002], especially dysplastic sporadic ACF [75% (3 of 4) versus 8% (2 of 24), *P* = 0.004]. MINT31 was more frequently methylated in heteroplastic ACF than dysplastic ACF [35% (11 of 31) versus 7% (2 of 30), *P* = 0.01]. Strong associations of ACF methylation with *K-ras* mutation (*P* = 0.007) and with loss of chromosome 1p (*P* = 0.04) were observed, but methylation was the only molecular abnormality identified in 16% (10 of 61) of ACF. Our findings suggest that methylation in ACF is an early event in the pathogenesis of a subset of colorectal carcinomas, and that ACF from FAP patients and patients with sporadic colorectal cancer have distinct epigenetic changes that reflect differences in molecular pathogenesis. (*Am J Pathol* 2002, 160:1823-1830)**

Aberrant crypt foci (ACF) in colorectal mucosa are the earliest known morphological precursors to colorectal cancer (CRC).<sup>1-6</sup> A role for ACF in colorectal carcinogenesis is supported by the presence of histopathological intraepithelial neoplasia (dysplasia) in some ACF, and the expression of markers of proliferation and of tumor-associated antigens and lectin-binding moieties.<sup>1,2</sup> This role is further corroborated by the presence in some ACF of

genetic alterations that are present in colorectal carcinomas, such as alterations in the *adenomatous polyposis coli* (*APC*) tumor suppressor gene, *K-ras* proto-oncogene mutations, and microsatellite instability (MSI).<sup>1-6</sup>

The histopathology of human ACF is variable but can be subclassified into two broad categories: dysplastic and heteroplastic.<sup>1</sup> Dysplastic ACF resemble adenomas and are more common in familial adenomatous polyposis (FAP), which is because of germline mutation of the *APC* gene, than in patients with sporadic colorectal neoplasia. In addition to dysplasia, these ACF are characterized by abnormal epithelial proliferation in the upper aspects of the crypts, lack of *K-ras* mutations, and presence of *APC* mutations in dysplastic ACF from FAP patients but not patients with sporadic CRCs.<sup>5-6</sup> In contrast, heteroplastic ACF resemble hyperplastic polyps histopathologically, lack dysplasia, have proliferation mainly in the lower aspects of the crypts, have frequent *K-ras* mutations, and lack *APC* mutations.<sup>1,5,6</sup> Thus, ACF are phenotypically and genotypically diverse, but reflect their molecular pathogenesis.

Recent studies have shown that methylation of CpG islands is a molecular defect common in CRC.<sup>7</sup> 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.<sup>8</sup> CpG island methylation (CIM) is a mechanism for suppression of transcription of genes in physiological and pathological settings including neoplasia.<sup>9</sup> The recently discovered CpG island methylator phenotype (CIMP) is a novel pathway characterized by methylation of multiple CpG islands in colorectal carcinomas and adenomas, including genes known to be important in tumorigenesis such as the *p16* tumor suppressor gene and *hMLH1* mismatch repair gene.<sup>10,11</sup> In addition, CIMP is associated with *K-ras* mutations through methylation of *MGMT* (*O*<sup>6</sup>-methylguanine-DNA methyltransferase).<sup>12</sup>

Dysplastic ACF are recognized precursors to CRC, but the relationship of heteroplastic ACF is less certain.<sup>1-6</sup> However, recent studies have proposed a *hyperplastic polyp/serrated adenoma-carcinoma* sequence as an alternative pathway to the usual *adenoma-carcinoma* se-

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quence.<sup>13-17</sup> In addition, CIMP is present in hyperplastic polyps from patients with hyperplastic polyposis and colorectal neoplasia.<sup>18</sup> Therefore in the present study, we characterized ACF for the presence of CIM and compared the findings with histopathological and molecular alterations of importance in colorectal tumorigenesis, including *K-ras* mutation, loss of heterozygosity (LOH) of chromosome 1p, and MSI.

## Materials and Methods

### Specimens and Patients

ACF were isolated from the grossly normal mucosa in 10 colectomy specimens from patients with sporadic CRCs and from the nonpolypoid mucosa in 2 colectomy specimens from FAP patients with numerous polyps but no cancer. The specimens were collected in surgical pathology at the M. D. Anderson Cancer Center. The colons or rectums were opened longitudinally and rinsed of their luminal contents. The mucosa was dissected from the underlying muscularis propria, and the entire mucosal sheet was pinned flat on wax and fixed in formalin for 2 to 4 hours. The fixed colonic sheet was stained with 0.2% methylene blue for several minutes, and the ACF were visualized using an Olympus dissecting microscope (Olympus Optical Company, Ltd., Tokyo, Japan). ACF were marked with India ink, excised from the mucosal sheet in small strips, and submitted for routine histological processing. The mucosal strips were embedded longitudinally in paraffin blocks, sectioned onto glass slides, and stained with hematoxylin and eosin (H&E).

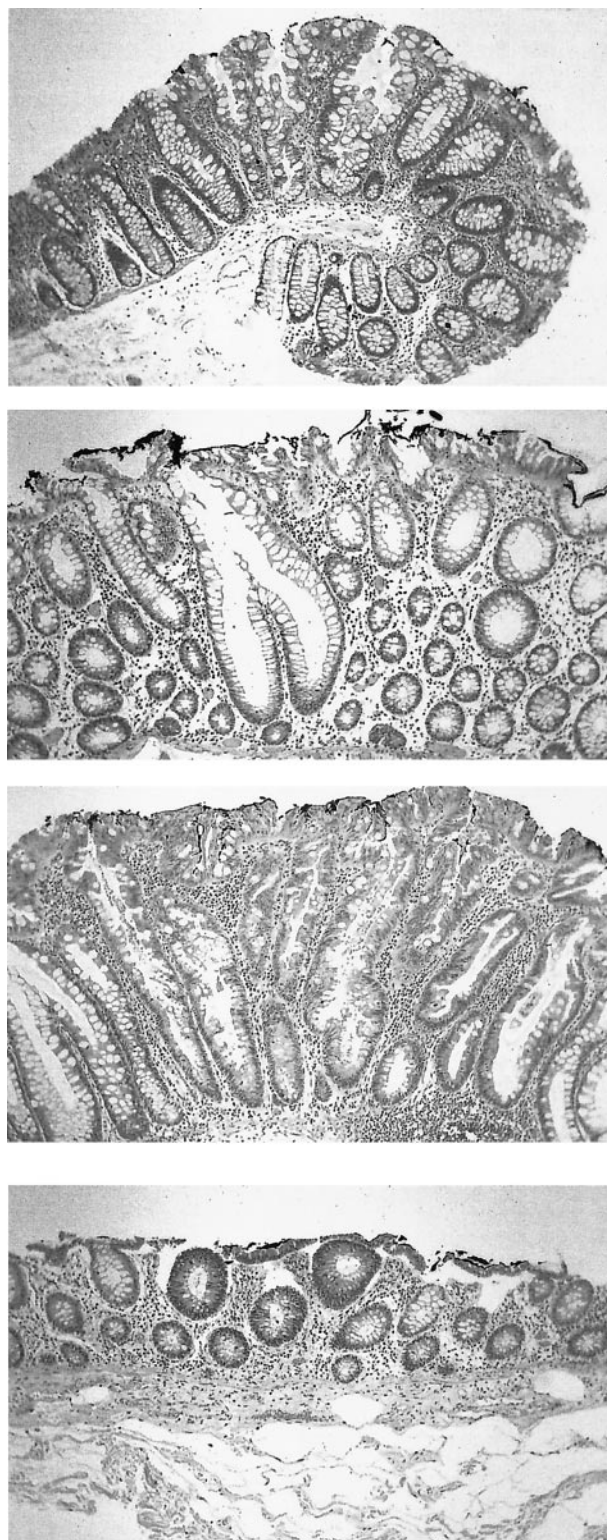
The H&E-stained ACF were characterized by light microscopy (Figure 1) of coded specimens by two gastrointestinal pathologists (RB and AR) unaware of the source of the specimens (Figure 1). The ACF were classified as dysplastic, heteroplastic, or mixed (features of both dysplastic and heteroplastic ACF).<sup>1</sup> The size of ACF was measured from the H&E-stained slides.

### DNA Extraction

Lesional and nonlesional tissues were microdissected from the H&E-stained sections of ACF and mucosa without coverslip. Genomic DNA was extracted and prepared from the microdissected ACF as described previously.<sup>19</sup>

### Bisulfite Treatment of DNA and Methylation-Specific PCR

The methylation status of the *p16* tumor suppressor gene, MINT1 (methylated in tumor 1), MINT2, MINT31, *MGMT* gene, and *hMLH1* mismatch repair gene was determined by bisulfite treatment of DNA followed by methylation-specific PCR as described.<sup>12,20</sup> The six loci selected for methylation analysis are unmethylated (<1% methylation) in normal tissues.<sup>7</sup> MINT1 and MINT2 correspond to CpG islands that are in the 5' region of cDNAs with



**Figure 1.** Histopathology of ACF. Heteroplastic ACF (top two panels), mixed heteroplastic and dysplastic ACF (third panel), and dysplastic ACF (bottom panel).

open reading frames that have no known protein homology (JP Issa, unpublished data). MINT31 is 2-kb upstream of the *CACNA1G*, a T-type calcium channel gene.<sup>21</sup>

**Table 1.** Primers and PCR Conditions for MINT 1, MINT 2, MINT 31, *MGMT*, and *bMLH1*

Locus	Allele	Sense primers	Antisense primers	Cycling conditions
MINT1	Methylated	5'-AATTTTTTTATATATATTTTCGAAGC-3'	5'-AAAAACCTCAACCCCGC-3'	95°C for 10 minutes, 37 cycles of
	Unmethylated	5'-AATTTTTTTATATATATTTTGAAGTGT-3'	5'-AACAAAAACCTCAACCCACA-3'	95°C for 30 seconds, 55°C for 45 seconds
MINT2	Methylated	5'-TTGTTAAAGTGTGAGTTCGTC-3'	5'-AATAACGACGATTCCGTACG-3'	95°C for 10 minutes, 40 cycles of
	Unmethylated	5'-GATTTTGTAAAGTGTGAGTTTGT-3'	5'-CAAATAATAACAACAATCCATACA-3'	95°C for 30 seconds, 60°C for 45 seconds
MINT31	Methylated	5'-TGTGGGGAAGTGTTCGCG-3'	5'-CGAAAACGAAACCCCGC-3'	95°C for 10 minutes, 38 cycles of
	Unmethylated	5'-TAGATGTGGGGAAGTGTTCGCG-3'	5'-TAAATACCCAAAAACAAACACCACA-3'	95°C for 30 seconds, 60°C for 45 seconds
MGMT	Methylated	5'-GGTCGTTGTACGTTTCGC-3'	5'-GACCGATACAAACCGAACG-3'	95°C for 10 minutes, 38 cycles of
	Unmethylated	5'-GTAGGTTGTTGTATGTTGT-3'	5'-AACCAATACAAACCAACA-3'	95°C for 30 seconds, 55°C for 45 seconds
hMLH1	Methylated	5'-GATAGCGATTTTAAACGC-3'	5'-TCTATAAATTACTAAATCTCTTCG-3'	95°C for 10 minutes, 40 cycles of 95°C for 30 seconds, 53°C for 45 seconds
	Unmethylated	5'-AGAGTGGATAGTGATTTTAAATGT-3'	5'-ACTCTATAAATTACTAAATCTCTTCA-3'	

In brief, 2  $\mu$ g of microdissected genomic DNA were denatured with 2 mol/L of NaOH at 37°C for 10 minutes, followed by incubation with 3 mol/L of sodium bisulfite (pH 5.0) at 50°C for 16 hours in darkness. After treatment, DNA was 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 ammonium acetate and 100% ethanol, washed with 70% ethanol, and finally resuspended in 20  $\mu$ l of distilled water.

The primers and polymerase chain reaction (PCR) conditions for *p16* were the same as reported by Herman and colleagues.<sup>20</sup> The primers and PCR conditions for the MINT loci, *MGMT*, and *hMLH1* are listed in Table 1. In brief, 2  $\mu$ l of bisulfite-treated DNA was used as template for PCR reactions using primers specific for methylated and unmethylated alleles. DNA from the RKO colon cancer cell line (American Type Culture Collection, Manassas, VA) and water were used as positive and negative controls, respectively. PCR products from methylated and unmethylated reactions were electrophoresed on 6% acrylamide gels and visualized by ethidium bromide staining.

### Mutation of *K-ras* Proto-Oncogene

The first exon of *K-ras* was amplified in 50- $\mu$ l volumes using 2  $\mu$ l of genomic DNA, 10 mmol/L Tris-HCl, pH 8.3, 50 mmol/L potassium chloride, 2 mmol/L magnesium chloride, 0.8 mmol/L dNTP mix, 2.25 U Ampli *Taq* Gold (Applied Biosystems, Foster City, CA), 0.125 U pfu DNA Polymerase (Stratagene, La Jolla, CA), and 20 pmol of forward and reverse primers (5'-GGCCGGTAGTGTATTAACCTTATG TGTGACAT-3' and 5'-CCGCGGCCG-GCGCCAAAACAAGATTTACCTCTATTGTTGG-3'; Life Technologies, Rockville, MD). PCR reactions were performed using the following cycling conditions: denaturation at 95°C for 10 minutes; 14 cycles of 95°C for 20

seconds, 59°C to 52°C in 0.5°C decrements/cycle for 60 seconds, and 72°C for 60 seconds; 25 cycles of 95°C for 20 seconds, 52°C for 60 seconds, and 72°C for 60 seconds; and extension at 72°C for 10 minutes on a GeneAmp PCR System 9700 (Applied Biosystems).

PCR products were purified using 10 U of exonuclease I and 2 U of shrimp alkaline phosphatase (Amersham Life Science, Indianapolis, IN), incubated at 37°C for 15 minutes, and inactivated by incubating at 80°C for 15 minutes. DNA sequencing was performed in 20- $\mu$ l volumes comprised of 2  $\mu$ l of purified PCR product, 8  $\mu$ l ABI Prism BigDye Terminator Cycle Sequencing Kit (Applied Biosystems), and 5 pmol of forward primer using the following cycling conditions: 25 cycles of 95°C for 20 seconds, 52°C for 60 seconds, and 72°C for 60 seconds. After spin-column purification (Princeton Separations, Adelphia, NJ), the reaction products were sequenced by capillary electrophoresis using an ABI Prism 3700 DNA Analyzer (Applied Biosystems). Mutations were confirmed by sequencing using the reverse primer.

### Fluorescently Labeled PCR Amplification

LOH and MSI were determined by fluorescently labeled PCR amplification using fluorescent dye-labeled and unlabeled primers (Applied Biosystems). The 5' oligonucleotide was end-labeled with 6-FAM (BAT-25, D1S199, and D1S507), HEX (BAT-26, D1S468, and D1S255), or NED (D1S214) fluorescent dye. PCR was performed in 15- $\mu$ l reaction volumes containing 40 ng of DNA, 9  $\mu$ l ABI Prism True Allele PCR Premix (Applied Biosystems), and 5 pmol of each primer. PCR was performed using the following cycling conditions: denaturation at 95°C for 12 minutes; 10 cycles (94°C for 15 seconds, 55°C for 15 seconds, 72°C for 30 seconds), 32 cycles (89°C for 15 seconds, 55°C for 15 seconds, 72°C for 30 seconds), and extension at 72°C for 10 minutes. A 1.0- $\mu$ l aliquot of each fluorescent-labeled PCR product was combined with 12



**Table 2.** Demographic Data and Characteristics of Patients with ACF

Patient	Age	Sex	Site of ACF	Histology of ACF, no.			Other clinical associations
				Heteroplastic	Dysplastic	Mixed	
1	50	F	L	2	7	0	FAP
2	17	M	R	1	17	0	FAP
3	49	M	L	7	0	0	Sporadic CRC
4	79	F	R	1	0	0	Sporadic CRC
5	69	M	R	0	0	1	Sporadic CRC
6	61	M	L	8	0	1	Sporadic CRC
7	64	F	L	3	1	0	Sporadic CRC
8	65	F	L	2	1	0	Sporadic CRC
9	37	F	L	2	0	0	Sporadic CRC
10	45	F	L	5	0	0	Sporadic CRC
11	60	F	L	0	1	0	Sporadic CRC
12	58	M	R	0	1	0	Sporadic CRC

F, female; M, male; L, left, colorectum; R, right, colon.

$\mu$ l of formamide and 0.5  $\mu$ l of Genescan 400HD (ROX) size standard (Applied Biosystems). The samples were then subjected to capillary electrophoresis on an ABI 3700 DNA Analyzer using Genescan Analysis software (Applied Biosystems).

### LOH of Chromosome 1p

Loss of chromosome 1p was determined by using five dinucleotide-repeat microsatellite markers on the short arm of chromosome 1 (in order from centromere to telomere D1S255, D1S199, D1S507, D1S214, and D1S468). Complete or partial loss of chromosome 1p was based on the pattern of loss of the five dinucleotide microsatellite markers evaluated. Loss of a marker was considered to be present when the PCR assay showed absence or decrease in intensity by more than 50% of a band from a tumor sample as compared with the paired control non-neoplastic sample.

### MSI

MSI status was determined by using the five markers on the short arm of chromosome 1p and two mononucleotide-repeat microsatellite markers, BAT 25 and BAT 26.<sup>13</sup> Specimens with high levels of MSI (MSI-high) were defined by shifts of bands as compared to control DNA in at least 30% of evaluable markers, and low levels of MSI (MSI-low) by shifts in less than 30% of evaluable microsatellite markers, as in previous studies.<sup>13</sup>

### Statistical Analysis

Fisher's exact test and chi-square test were used for comparing associations among genetic alterations and between genetic alterations and clinicopathological factors. Student's *t*-test was used to compare the means of size, age, and methylation.

### Results

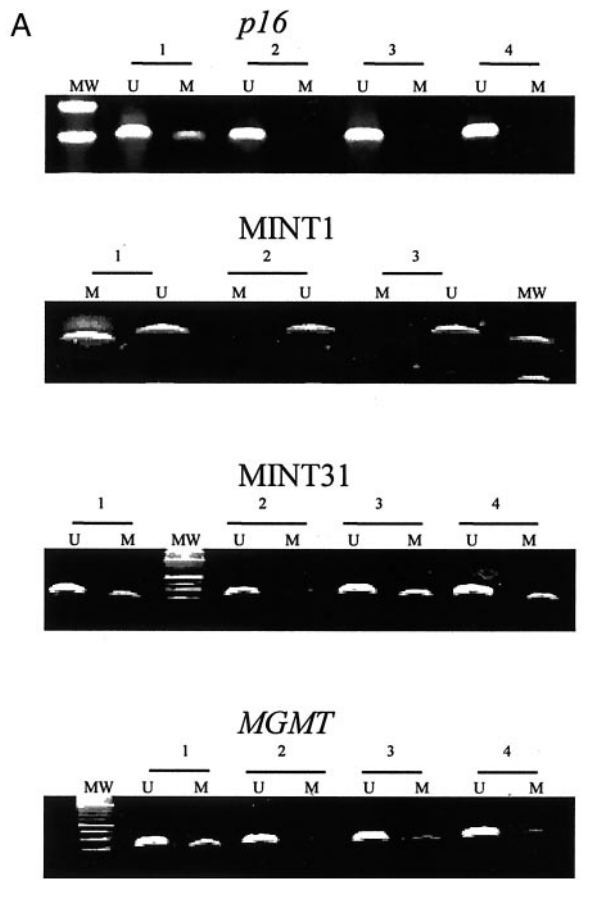
The patient demographic data in relationship to ACF characteristics are summarized in Table 2. Twenty-seven ACF were identified from two FAP patients and 34 ACF from 10 patients with sporadic colorectal carcinomas. The histopathological type of ACF was strongly associated with the patient groups: 89% (24 of 27) of ACF from FAP patients were dysplastic, and only 11% (3 of 27) were heteroplastic. In contrast, 82% (28 of 34) of ACF from the patients with sporadic CRC were heteroplastic, only 12% (4 of 34) were dysplastic, and 6% (2 of 34) were mixed having both dysplastic and heteroplastic regions ( $P = 0.000001$  for sporadic versus FAP patients).

### CpG Island Methylation in ACF

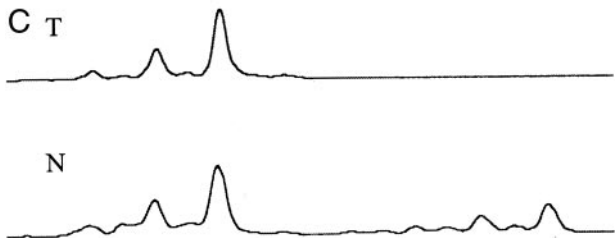
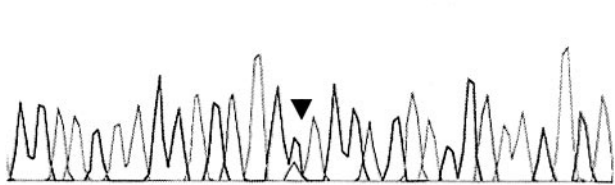
Methylation of the *p16* gene, MINT31, MINT2, MINT1, *MGMT* gene, and *hMLH1* gene was present in 4% (2 of 56), 21% (13 of 61), 5% (3 of 59), 8% (5 of 59), 12% (6 of 51), and 3% (2 of 61) of the ACF, respectively (examples in Figure 2A and summarized in Figure 3). Thirty-four percent (21 of 61) of the ACF were methylated in at least one locus: 2% (1 of 61) of ACF were methylated at three loci, 13% (8 of 61) at two loci, and 20% (12 of 61) at one locus. The *p16* gene was methylated in two ACF, the *hMLH1* gene in two ACF, and the *MGMT* gene in six ACF (Figure 3). Six of eight ACF with methylation of *p16*, *hMLH1*, or *MGMT* had methylation of at least one additional locus.

### CpG Island Methylation in Mucosa

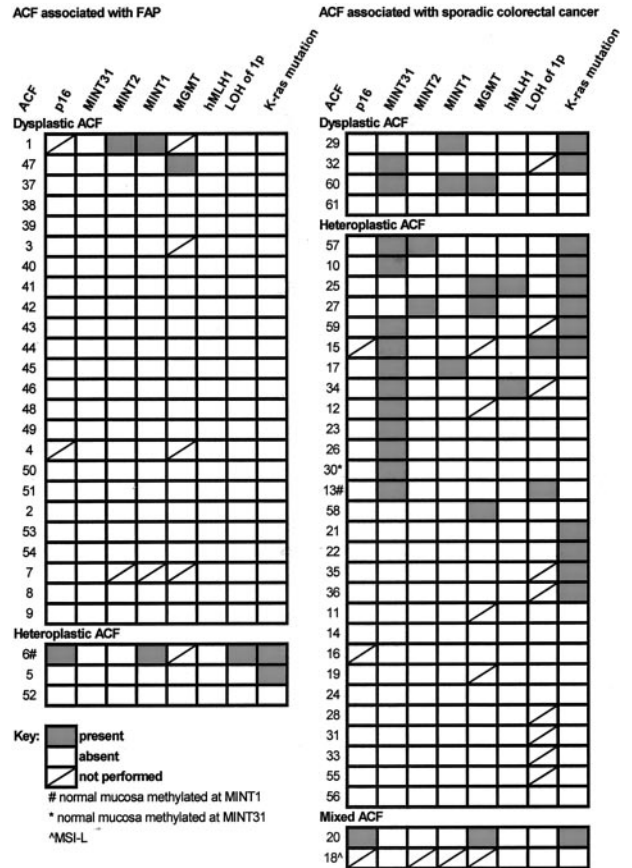
Methylation of the six markers was analyzed in 36 samples of mucosa adjacent to the ACF (Figure 3). Methylation at one locus was found in only three mucosal samples. Two of these samples were methylated at MINT31 and MINT1, respectively, and the adjacent ACF were concordantly methylated for the same loci. The third sample of mucosa was methylated at MINT1, and was discordant with the methylation status of the adjacent ACF.



**B**  
 GGTAGTTGGAGCTGGTGGC GTAGGCAAGAGT  
 70 80 90



**Figure 2. A:** Methylation analysis of CpG islands in ACF. Examples of methylation of *p16*, MINT1, MINT31, and *MGMT* are shown. Methylation-specific PCR using primers for methylated (M) and unmethylated (U) alleles of bisulfite-treated DNA was performed. Loci examined and ACF numbers are indicated above each gel. MW represents lane with molecular weight marker. **B:** Nucleotide sequencing of the *K-ras* gene in ACF. A G-to-A mutation of codon 12 is indicated by the **arrowhead**. **C:** Allelic loss of chromosome 1p in ACF. The lane from normal DNA (N) has two alleles at marker D1S199 and the lane from the tumor DNA (T) shows loss of one of the alleles.



**Figure 3.** CIM, loss of chromosome 1p, K-ras mutation, and histopathology of ACF from FAP patients and patients with sporadic CRC.

### *K-ras Mutation, Loss of Chromosome 1p, and MSI in ACF*

*K-ras* mutations were present in 25% (15 of 61) of ACF (examples in Figure 2B and summarized in Figure 3). All 15 mutations were present at codon 12, and none were found in codon 13. G-to-A transition at the second nucleotide of codon 12 was present in seven ACF, G-to-T transversion at the second nucleotide in seven ACF, and G-to-T transversion at the first nucleotide in one ACF.

Chromosome 1p loss was present in 6% (3 of 52) of ACF (examples in Figure 2C and summarized in Figure 3). Chromosome 1p loss was present at locus 1p35 (marker D1S199) in one ACF, and at locus 1p35-36 (markers D1S468 and D1S507, and markers D1S199 and D1S468, respectively) in the other two ACF.

Allelic shift was present in only one ACF, which was classified as MSI-low. No ACF had MSI-high.

### *Associations of CpG Island Methylation with Clinicopathological and Molecular Characteristics*

CIM occurred in ACF from FAP and sporadic patients but was more characteristic of sporadic ACF: methylation was present in 53% (18 of 34) of ACF from patients with

sporadic colorectal carcinomas but only 11% (3 of 27) of ACF from FAP patients ( $P = 0.002$ , Figure 3). This difference in methylation frequency was most apparent in dysplastic ACF, which had a higher frequency of methylation in sporadic patients than in FAP patients [75% (3 of 4) versus 8% (2 of 24),  $P = 0.004$ ]. In contrast, the frequency of methylation was similar in heteroplasic ACF from FAP and sporadic patients [33% (1 of 3) versus 50% (14 of 28);  $P = 0.9$ , not significant].

When individual markers were considered, MINT31 was more frequently methylated in heteroplasic ACF than dysplastic ACF [35% (11 of 31) versus 7% (2 of 30),  $P = 0.01$ ], but the other methylation sites had no significant difference between the histopathological types. The *p16* gene was methylated in one heteroplasic ACF from an FAP patient and in a mixed ACF from a patient with sporadic colon cancer. Methylation of the *hMLH1* gene was present in two microsatellite-stable heteroplasic ACF from two patients with sporadic CRC (Figure 3). The *MGMT* gene was methylated in six ACF (Figure 3), including dysplastic ACF from FAP and sporadic patients and heteroplasic ACF from sporadic patients. Patient age, and site and size of ACF were not associated with the methylation status of the ACF.

*K-ras* mutation was more common in ACF from sporadic than FAP patients but was found in dysplastic ACF, whereas LOH of chromosome 1p was found only in heteroplasic ACF. *K-ras* mutation was present in 7% (2 of 27) of ACF from FAP patients but in 38% (13 of 34) of ACF from patients with sporadic carcinomas ( $P = 0.01$ ). Mutation was present in 10% (3 of 30) of ACF with dysplasia, but 39% (12 of 31) of heteroplasic ACF ( $P = 0.02$ ). Loss of 1p was present in 13% (3 of 23) of heteroplasic ACF, consisting of one ACF from an FAP patient and two ACF from two patients with sporadic CRC, but in none of 29 dysplastic or mixed ACF.

*K-ras* mutations and LOH of chromosome 1p were more common in ACF with CIM. *K-ras* mutation was present in 48% (10 of 21) of ACF with CIM, but only 13% (5 of 40) of ACF without methylation ( $P = 0.007$ ). Two of three ACF with *K-ras* mutations and methylation of *MGMT* had G-to-A transition, as reported in a previous study of adenomas or carcinomas.<sup>12</sup> Similarly, chromosome 1p loss was present in 20% (3 of 18) of ACF with CIM, but in 0 of 34 ACF without methylation ( $P = 0.04$ ).

When the clinicopathological and more frequent molecular markers were considered together, 92% (22 of 24) of dysplastic ACF from FAP patients lacked methylation or *K-ras* mutation (dysplasia +, methylation -, *K-ras* mutation -; Figure 3). In contrast, the heteroplasic, mixed, and dysplastic sporadic ACF and the heteroplasic ACF from FAP patients were much more heterogeneous than dysplastic FAP ACF: 35% ( $n = 13$ ) were without methylation or *K-ras* mutation (methylation -, *K-ras* mutation -); 24% ( $n = 9$ ) had methylation (methylation +, *K-ras* mutation -); 27% ( $n = 10$ ) had both methylation and *K-ras* mutation (methylation +, *K-ras* mutation +); and 14% ( $n = 5$ ) had *K-ras* mutation (methylation -, *K-ras* mutation +).

## Discussion

The role of ACF in colorectal carcinogenesis has been contentious, and ACF could be precursors or innocent bystanders.<sup>1-6</sup> The *dysplastic ACF-adenoma-carcinoma sequence* is well-recognized because dysplastic ACF are frequent in FAP patients, and *APC* mutations are present in dysplastic ACF from FAP patients and a minority of sporadic dysplastic ACF.<sup>5,6,22</sup> The role of ACF as precursors of some CRCs is further corroborated by the presence of MSI in ACF from patients with hereditary non-polyposis CRC in whom carcinoma with high levels of MSI is the hallmark, and in ACF from sporadic patients.<sup>3,23</sup> The role of heteroplasic ACF in colorectal carcinogenesis is more controversial because these ACF have frequent *K-ras* mutation but lack dysplasia or *APC* mutations, in contrast to most adenomas and carcinomas.<sup>5,6,22</sup> However, a role for heteroplasic ACF as a precursor to a subset of CRCs is supported by the following lines of evidence: heteroplasic ACF are clonal;<sup>24</sup> genetic alterations that are common in CRCs such as *K-ras* mutation,<sup>1,2,4-6,22</sup> chromosome 1p loss (the present study), and/or CIM (the present study) are present in heteroplasic ACF; ACF, adenomas, and carcinomas share similar incidence and anatomical distribution;<sup>25-27</sup> and ACF with mixed heteroplasic and dysplastic components exist.<sup>28</sup> A *heteroplasic ACF-adenoma-carcinoma sequence* has been proposed in which *K-ras* mutation precedes *APC* mutation in human and rodent studies.<sup>6,29</sup>

An alternative pathway of colorectal carcinogenesis with a *serrated polyp-dysplasia-adenocarcinoma sequence* has also been proposed.<sup>13-17</sup> Jass<sup>17</sup> has reported in colorectal carcinogenesis the importance of serrated lesions, low levels of MSI (MSI-L), and *ras* mutation. In a previous study, we have reported concordant methylation of multiple hyperplastic polyps in patients with hyperplastic polyposis and colorectal neoplasia including serrated adenomas.<sup>18</sup> Heteroplasic ACF therefore may be precursors of serrated lesions that progress to CRCs.

CIM of one or more loci was present in a third of ACF in our study, including both dysplastic and heteroplasic types in both FAP and sporadic patients. A previous study has reported methylation of the *androgen receptor* gene on the X chromosome in human ACF.<sup>24</sup> Because ACF are putative precursor lesions, these studies provide evidence that CIM is an early event in colorectal carcinogenesis.

Previous studies have identified genetic alterations in ACF, including *APC* gene alterations, *K-ras* mutations, and MSI.<sup>1-6,22</sup> We also found loss of chromosome 1p in 6% of ACF, and previous studies have identified chromosome 1p loss in colorectal hyperplastic polyps, adenomas, and carcinomas.<sup>13,30,31</sup> Our study therefore broadens the spectrum of molecular abnormalities that are identified in ACF as well as in tumors.

Methylation at multiple loci is present in 40 to 50% of sporadic colorectal carcinomas and adenomas,<sup>7,10,11</sup> and in hyperplastic polyps from patients with hyperplastic polyposis.<sup>18</sup> Methylation of two or more loci was present in 15% of ACF in our study, and methylation of

genes known to be important in tumorigenesis was associated with methylation of multiple loci. In our study, four of six ACF with *MGMT* methylation, both ACF with *hMLH1* methylation, and the one ACF with p16 methylation were methylated at more than one locus. CIM in ACF was associated with *K-ras* mutation, and G-to-A transition was present in three ACF with methylation of *MGMT* in our study. A previous study has shown an association between CIM in cancer and *K-ras* mutation,<sup>10</sup> and another previous study reported an association between G-to-A transition in *K-ras* gene and methylation of *MGMT* in colorectal carcinomas and large adenomas.<sup>12</sup> These findings suggest that CIM of *MGMT* is an early event and precedes the G-to-A transition mutations of the *K-ras* gene. Similarly, methylation of the *hMLH1* mismatch repair gene in ACF was not associated with the development of MSI, suggesting that *hMLH1* methylation precedes MSI. These results emphasize the early occurrence of abnormal methylation as a pathogenic mechanism in colorectal carcinogenesis.

In our study, the frequency of CIM was related to the type of patient with ACF: methylation was present in 53% of ACF from patients with sporadic colorectal carcinomas but only 11% of ACF from FAP patients. Of note, methylation was more frequent in dysplastic sporadic ACF than in dysplastic FAP ACF (75% versus 8%). Previous studies have shown differences in frequencies of dysplasia and *K-ras* mutation in ACF from patients with sporadic colorectal carcinomas and FAP patients.<sup>5,6,22</sup> ACF from patients with sporadic carcinomas usually had CIM and/or *K-ras* mutation and lacked dysplasia. In contrast, ACF from FAP patients usually lacked CIM or *K-ras* mutation but were frequently dysplastic, reflecting the inherited predisposition to dysplasia resulting from germline *APC* gene mutation. Our data on methylation provide additional evidence that phenotypic and genetic progression in FAP differs from some sporadic colorectal carcinomas. Dysplastic ACF occur in sporadic patients, although at low frequency, and have characteristics such as those of dysplastic ACF in FAP patients but with less frequent *APC* mutation,<sup>5,6</sup> and more frequent methylation in our study. In addition, we found that MINT31, which is present 2-kb upstream of the *CACNA1G*, a *T-type calcium channel* gene,<sup>21</sup> was differentially methylated in heteroplastic but not dysplastic ACF with higher frequency in the heteroplastic type.

In conclusion, the epigenetic differences in methylation in ACF we found are analogous to the heterogeneity of phenotypic and genetic characteristics in colorectal carcinomas. Understanding of the events in ACF provides the opportunity for their prevention and suppression of their progression to malignancy.

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