Epigenetic alterations in RASSF1A in human aberrant crypt foci

Emily J.Greenspan1, Melissa A.Jablonski1, Thiruchandurai V.Rajan2,3, Joel Levine2,4, Glenn S.Belinsky1 and Daniel W.Rosenberg1,2,*

1Center for Molecular Medicine, 2Colon Cancer Prevention Program, Neag Comprehensive Cancer Center, 3Department of Pathology and 4Division of Gastroenterology, UCHC School of Medicine, University of Connecticut Health Center, Farmington, CT, USA

*To whom correspondence should be addressed at: University of Connecticut Health Center, Center for Molecular Medicine, 263 Farmington Avenue, Farmington, CT 06030-3101. Tel: +860 679 8704; Fax: +860 679 7639; Email: Rosenberg@ns01.uchc.edu

Introduction

Aberrant crypt foci (ACF) are microscopic surface abnormalities that form the basis for the adenoma-carcinoma sequence in CRC (4,5). Genetic alterations in ACF include mutations in the APC tumor suppressor gene and the K-ras proto-oncogene, and microsatellite instability (MSI) (5–8). With the advent of new endoscopic instrumentation, it is possible to visualize ACF in situ at high resolution using colonoscopy (4,9). The histopathology of ACF is variable, but is generally classified into two major categories: hyperplastic and dysplastic (Figure 1) (10). Hyperplastic ACF display characteristics similar to hyperplastic polyps and to adenomas and are more common in sporadic cases than in FAP patients (3,4). They are characterized by frequent K-ras mutations and lack APC mutations (7,8). On the other hand, dysplastic ACF display abnormal proliferation within upper regions of the crypts and, in sporadic cases, generally have mutations in K-ras and lack APC mutations, although dysplastic ACF from FAP patients have frequent APC mutations (7,8). While dysplastic ACF are generally accepted as precursors to CRC, the significance of hyperplastic ACF in tumor progression is less well established (3,4). Therefore, it is important to fully characterize the genetic alterations that are present in hyperplastic ACF to better understand the distinct molecular pathways leading to CRC.

Epigenetic silencing caused by DNA CpG island methylation (CIM) is a mechanism for transcriptional silencing (11). CIM has been identified during several key stages in colon tumorigenesis, including ACF, sporadic serrated adenomas, hyperplastic polyps and tumors (12–16). In sporadic cases of CRC, studies have proposed that DNA hypermethylation mediates a ‘field defect’ whereby large regions of colonic epithelium have sustained genetic aberrations (17,18). In CRC, a panel of genes that have been shown to be silenced by promoter hypermethylation include p16, hMLH1, MGMT, and MINT 1, 2, 12 and 31 (14–16). Carcinomas and adenomas that are methylated at multiple loci (two or more) are referred to as having the CpG island methylator phenotype high status (CIMP high) (14–16).

RASSF1A is a putative tumor suppressor gene located at chromosome 3p21.3, a region that frequently exhibits loss of heterozygosity in human tumors. RASSF1A is a major isoform of the RASSF1 gene that is generated by alternative splicing. The C-terminus of RASSF1 encodes a Ras association domain. RASSF1A transcripts are often missing in cancer cell lines and in tumors (19,20). In addition, RASSF1A knockout mice show increased tumor multiplicity and size (21). In sporadic CRC, reported RASSF1A inactivation through promoter hypermethylation is variable (~30% of cases) (22–24). Since RASSF1A and K-ras share a common signaling pathway, it has been proposed that hypermethylation of RASSF1A and mutations in K-ras are mutually exclusive (23–25). However, more recent data suggest that RASSF1A inactivation and K-ras mutations are concurrent in MSI sporadic CRC, suggesting a synergistic effect (22).

In the following study, ACF were identified in and biopsied in situ from patients at elevated risk for CRC using a prototype
Materials and methods

Subject selection
All patients included in this study were considered to be at elevated risk for CRC based on a positive family history (at least one first- or second-degree relative with the disease) and/or a positive personal history for benign polyps/adenomas. All patients meeting the Amsterdam criteria for HNPCC or FAP were excluded from this study. No subjects used in this study had a history of prior malignancy or had evidence of a synchronous colon tumor(s). Patients underwent total colonoscopy at the John Dempsey Hospital (JDH) at the University of Connecticut Health Center (UCHC) in accordance with institutional policies. This study was performed after approval by an Institutional Review Board and all subjects provided written informed consent.

ACF collection and characterization
ACF were isolated from grossly normal appearing colonic mucosa by biopsy in situ during the high-resolution close-focus chromendoscopy portion of total colonoscopy procedures performed in the JDH at UCHC. The cecum and, in addition, 20 cm of the distal colon (encompassing part rectum, part sigmoid colon) were stained with up to 40 ml of 0.5% methylene blue to identify ACF. ACF were visualized and photographed using an Olympus Prototype Close Focus Colonoscope (XCF-Q160ALE), which enables visualization from 2 to 100 mm at a magnifying power of ×60 (Figure 1A) (4). Under close-focus magnification, a finding was accepted as an ACF if two or more crypts had increased lumen diameter, thick crypt walls or abnormally shaped lumens. Biopsies of individual ACF were embedded immediately in tissue freezing medium (OCT) and stored at −80°C. Biopsies of ACF directly abutting the aberrant crypts were collected separately by laser capture.

DNA and RNA extraction
Frozen sections of ACF were prepared and subjected to sequencing using the ABI Prism BigDye Terminator (Applied Biosystems, Foster City, CA), 2.0 μl 5× BigDye reaction buffer (Applied Biosystems) and 3.2 pmoles forward primer. Reaction cycling conditions were as follows: 96°C for 2 min, 28 cycles of 96°C for 10 s, 50°C for 5 s and 72°C for 4 min. The reaction products were sequenced by capillary electrophoresis using an ABI Prism 3100 DNA Analyzer. Mutations were confirmed by sequencing using the reverse primer. HCT116 cells were used as a positive control for a mutation in codon 13, and HEK293 cells were used as a negative control.

RASSF1A methylation-specific PCR (MSP)
The methylation status of RASSF1A was determined by sodium bisulfite treatment of DNA (27) followed by MSP (24). Briefly, 1 μg of salmon sperm DNA was added as a carrier to 10–30 ng of microdissected genomic DNA, or 100 ng of genomic DNA from cell lines, and the total sample volume was brought to 50 μl with nuclease-free H2O. DNA was denatured with 7.5 μl of 2M NaOH at 37°C for 10 min. DNA was incubated with 3 μl of 100 mM hydroquinone and 540 μl of 3M sodium bisulfite (pH 5.0) at 50–55°C for 16 h in darkness. After treatment, DNA was purified using the Wizard DNA Cleanup Kit (Promega, Madison, WI) and de-sulfonated with 4.4 μl of 3M NaOH at 37°C for 15 min. DNA was precipitated with 1/10 volume of 3M sodium acetate (pH 5.2) and 3 volumes of 100% ethanol, washed with 70% ethanol and resuspended in 25 μl nuclease-free water.

MSP primers were the same as those reported by van Engeland et al. (24). Nested PCR was performed to increase reaction sensitivity. A 144 bp product was amplified in 25 μl volumes using 6 μl bisulfite-treated genomic DNA, 10x PCR buffer, 50 mM MgCl₂, 10 mM dNTPs, 0.5 μl Platinum Taq and 2.5 pmoles forward and reverse primers. The primers used for the first round of PCR were 5'-GGG TTC GTT TGG TGT CGT TC-3' (sense) and 5'-GCC CTA ACT CAA TAA ACT CAA ACT-3' (antisense). PCR reactions were performed using the following cycling conditions: 95°C for 5 min, 23 cycles of 95°C for 45 s, 59°C for 45 s and 72°C for 1 min, and extension at 72°C for 10 min. The resulting 144 bp fragment was used as a template for the MSP reaction. 1:100 dilutions of the first PCR product were made, and the methylated and unmethylated products were amplified using the same reaction mixture as above. A 76 bp methylated product was amplified using the primers 5'-GGG TTC GTT TGG TGT CGT CT-3' (sense) and 5'-TAA CCC GAT TAA ACC CGT ACT TCG-3' (antisense). An 81 bp unmethylated product was amplified using the primers 5'-GGG GGT TTG TTG TTG TTG TTG TTG TTT TGG TTT TTT TGG TTT TTT TGG TTT TTT TGG TTT TTT TGG TTT TGT-3' (sense) and 5'-AAC ATA ACC CAA TTA AAC CCA TAC TTT A-3' (antisense). The cycling conditions were 95°C for 5 min, 30 cycles of 95°C for 45 s, 63°C (67°C for the methylated reaction) for 45 s and 72°C for 1 min, and 72°C for 10 min. All PCR reactions were performed with two controls for methylation (A549 and HEK293 cells), one control for no methylation (HCT116 cells) and a H₂O control. Twenty-five microliters of each PCR reaction was loaded onto a 2% agarose gel, stained with ethidium bromide and visualized under UV illumination.

RASSF1A mRNA expression
LCM was performed on frozen sections of ACF, and RNA was extracted as described above. Eleven microliters of microdissected RNA was reverse-transcribed to cDNA using a SensiScript RT Kit (Qiagen). cDNA was used as template in real-time PCR reactions. Specificity of PCR reactions was confirmed by sequencing using the ABI Prism BigDye reaction buffer and 3.2 pmoles forward primer. Cycling conditions were 94°C for 2 min, 50°C for 1 min, 40 cycles of 94°C for 15 s and 60°C for 1 min. Primer sequences were 5'-GCC TGC TGA AAA TCA CTG AA-3' (sense) and 5'-AGA AGT GTC CTG CAC CAG TAA-3' (antisense), and a 35-cycle PCR was performed using 58°C as the annealing temperature. PCR products were separated on a 2% agarose gel, excised and subjected to sequencing using the ABI Prism BigDye Terminator kit (Applied Biosystems, Foster City, CA), 2.0 μl 5× BigDye reaction buffer (Applied Biosystems) and 3.2 pmoles forward primer. Reaction cycling conditions were as follows: 96°C for 2 min, 28 cycles of 96°C for 10 s, 50°C for 5 s and 72°C for 4 min. The reaction products were sequenced by capillary electrophoresis using an ABI Prism 3100 DNA Analyzer. Mutations were confirmed by sequencing using the reverse primer. HCT116 cells were used as a positive control for a mutation in codon 13, and HEK293 cells were used as a negative control.

K-ras mutational analysis
Genomic DNA was extracted from microdissected tissue, precipitated with isopropanol and amplified using the GenomiPhi amplification kit (Amersham Biosciences, Piscataway, NJ). A 167 bp product spanning codons 12 and 13 of human K-ras was amplified in 25 μl volumes using 1 μl of amplified genomic DNA, 10X polymerase chain reaction (PCR) buffer, 50 mM MgCl₂, 10 mM dNTPs, 0.5 μl Platinum Taq and 2.5 pmoles forward and reverse primers (Invitrogen). Primers were designed using Primer3 software (5'-GCC TGC TGA AAA TCA CTG AA-3' (sense) and 5'-AGA AGT GTC CTG CAC CAG TAA-3' (antisense)), and a 35-cycle PCR was performed using 58°C as the annealing temperature. PCR products were separated on a 2% agarose gel, stained with ethidium bromide and visualized under UV illumination.

Statistical analysis
For comparison of K-ras mutations, RASSF1A hypermethylation and clinicopathological features, analysis was carried out for two-sided P-values using the χ²-test and Web Chi Squared Calculator (http://www.georgetown.edu/faculty/ballc/webtools/web_chi.html).

Results
The subjects included in this study did not have a past or current colon cancer. They were identified as being at elevated risk on the basis of a positive family history of CRC or a...
personal history of benign polyps or adenomas. As shown in Table I, 41.7% (5 out of 12) of subjects had a personal history of benign polyps or adenomas, 33.3% (4 out of 12) had a positive family history of CRC and 25% (3 out of 12) had a personal history of benign lesions as well as a family history of CRC. The patients were from 46 to 79 years of age (average = 63). All of the patients included in our study presented with multiple ACF at the time of colonoscopy (3–12 ACF each), with an average of 6 ACF per subject.

Of the total number of ACF identified during colonoscopy from all subjects, 56.3% (40 out of 71) displayed serrated crypts, normal nuclei and mucin depletion and were classified histologically as hyperplastic. Representative images of hyperplastic and dysplastic ACF collected during our study are shown in Figure 1. As shown in Figure 2, ACF were isolated from the surrounding colonic epithelium by LCM for (epi)genetic analysis. Whenever possible, ANM was captured separately by LCM (Figure 2). We assessed K-ras mutations in codons 12 and 13 by direct PCR sequencing. As shown in Table I, 8.6% (3 out of 35) of ACF were found to have a K-ras mutation in either codon 12 or codon 13 and were hyperplastic. A G-to-A transition mutation at the second nucleotide of codon 12 was present in two ACF and a G-to-C transversion at the second base of codon 13 was present in one ACF. Of the two dysplastic ACF examined, neither had K-ras mutations (Table I).

We measured RASSF1A promoter methylation in hyperplastic ACF and adjacent normal epithelium in 25 samples from 10 subjects by MSP. The locations of CpG sites and regions amplified by MSP primers within the RASSF1A promoter from the surrounding colonic epithelium by LCM for (epi)genetic analysis. Whenever possible, ANM was captured separately by LCM (Figure 2). We assessed K-ras mutations in codons 12 and 13 by direct PCR sequencing. As shown in Table I, 8.6% (3 out of 35) of ACF were found to have a K-ras mutation in either codon 12 or codon 13 and were hyperplastic. A G-to-A transition mutation at the second nucleotide of codon 12 was present in two ACF and a G-to-C transversion at the second base of codon 13 was present in one ACF. Of the two dysplastic ACF examined, neither had K-ras mutations (Table I).

We measured RASSF1A promoter methylation in hyperplastic ACF and adjacent normal epithelium in 25 samples from 10 subjects by MSP. The locations of CpG sites and regions amplified by MSP primers within the RASSF1A promoter

<table>
<thead>
<tr>
<th>Patient</th>
<th>Sex</th>
<th>Age</th>
<th>Total ACF</th>
<th>Sample number</th>
<th>ACF histology</th>
<th>K-ras mutation (codons 12 and 13)</th>
<th>RASSF1A methylation</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1&lt;sup&gt;b&lt;/sup&gt;</td>
<td>M</td>
<td>Unknown</td>
<td>5</td>
<td>042004-1130-3</td>
<td>Hyperplastic</td>
<td>WT</td>
<td>U&lt;sup&gt;s&lt;/sup&gt;</td>
</tr>
<tr>
<td>2&lt;sup&gt;b&lt;/sup&gt;</td>
<td>M</td>
<td>Unknown</td>
<td>5</td>
<td>030904-1130-5</td>
<td>Hyperplastic</td>
<td>WT</td>
<td>U</td>
</tr>
<tr>
<td>3&lt;sup&gt;b&lt;/sup&gt;</td>
<td>F</td>
<td>74</td>
<td>4</td>
<td>112003-1045-4</td>
<td>Hyperplastic</td>
<td>WT</td>
<td>U</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>4&lt;sup&gt;b&lt;/sup&gt;</td>
<td>M</td>
<td>49</td>
<td>6</td>
<td>021904-1000-2</td>
<td>Hyperplastic</td>
<td>WT</td>
<td>U</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>5&lt;sup&gt;a&lt;/sup&gt;,&lt;sup&gt;f&lt;/sup&gt;</td>
<td>F</td>
<td>50</td>
<td>3</td>
<td>121103-1100-4</td>
<td>Dysplastic</td>
<td>WT</td>
<td>U</td>
</tr>
<tr>
<td>6&lt;sup&gt;a&lt;/sup&gt;,&lt;sup&gt;f&lt;/sup&gt;</td>
<td>M</td>
<td>65</td>
<td>5</td>
<td>042604-1130-1</td>
<td>Hyp. &amp; dyspl.</td>
<td>WT</td>
<td>U</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>7&lt;sup&gt;a&lt;/sup&gt;</td>
<td>M</td>
<td>73</td>
<td>9</td>
<td>032904-230-2</td>
<td>Hyperplastic</td>
<td>WT</td>
<td>U</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>8&lt;sup&gt;a&lt;/sup&gt;</td>
<td>F</td>
<td>51</td>
<td>7</td>
<td>042904-1200-5</td>
<td>Hyperplastic</td>
<td>WT</td>
<td>U</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>9&lt;sup&gt;b&lt;/sup&gt;</td>
<td>M</td>
<td>Unknown</td>
<td>12</td>
<td>101804-1100-2</td>
<td>Hyperplastic</td>
<td>WT</td>
<td>U</td>
</tr>
</tbody>
</table>

All ACF assayed for mutations/methylation were located in the sigmoid colon. Total ACF refers to the number of ACF identified during the chromendoscopy procedure.

<sup>a</sup>ANM, adjacent normal mucosa.
<sup>b</sup>Personal history (past benign polyps/adenomas).
<sup>c</sup>U, unmethylated.
<sup>d</sup>HND, histology not determined.
<sup>e</sup>ND, none detected.
<sup>f</sup>Family history (at least one first- or second-degree relative with CRC).
<sup>g</sup>M, methylated.
<sup>h</sup>X, assay not performed.
Fig. 1. Macroscopic and histological analysis of ACF. (A) Gross views of ACF visualized through the Olympus prototype close-focus endoscope at a magnification of ×60. The colon epithelium was stained with 0.5% methylene blue. (B) H&E stained frozen sections of hyperplastic ACF, cross-sectional (left, ×200) and longitudinal (right, ×400) views. (C) H&E-stained frozen section of hyperplastic ACF with adjacent normal colonic mucosa indicated by the red arrows (×200). (D) H&E-stained frozen section of dysplastic ACF, cross-sectional view (×400). The inset in the upper left shows a region of surface dysplasia from the same lesion.

Fig. 2. Representative LCM of a hyperplastic ACF and adjacent normal colonic epithelium. LCM was used to isolate aberrant crypts in the hyperplastic ACF (right side, indicated by arrows) from the ANM (left side). The lower images show two populations of microdissected crypts isolated separately from the surrounding stroma. All images are at ×200 magnification.
region are detailed in Figure 3A. Figure 3B shows representative examples of the \textit{RASSF1A} MSP. Our study found that 24\% of ACF (6 out of 25) from 10 patients exhibited \textit{RASSF1A} methylation (Table I). In contrast, there was no evidence for \textit{RASSF1A} hypermethylation within LCM-isolated adjacent normal colonic mucosa (0 out of 6). Although the number of normal samples that were analyzed was too small to yield statistically significant results, these results indicate the absence of a methylation ‘field effect’ (Table I, Figure 3B).

To confirm that \textit{RASSF1A} hypermethylation is associated with decreased gene transcription, we measured the expression by QRT–PCR of \textit{RASSF1A} mRNA in three ACF exhibiting methylation, as well as two ACF that were unmethylated. Corresponding ANM was also evaluated. As shown in Figure 4, \textit{RASSF1A} expression ranged from 5.7 to 19.5-fold higher in adjacent non-methylated normal mucosa compared with the methylated ACF. In contrast, in the unmethylated ACF, \textit{RASSF1A} expression was not significantly different compared with the adjacent unmethylated normal mucosa, ranging from 0.75- to 2.16-fold higher. Expression was absent in HEK293 and A549 cells that are both fully methylated at the \textit{RASSF1A} promoter. However, HCT116 cells, which are characterized by an unmethylated \textit{RASSF1A} promoter, showed \textit{RASSF1A} expression (Figure 4).

**Discussion**

We have analyzed a subset of hyperplastic ACF identified by close-focus magnifying colonoscopy for \textit{RASSF1A}
methylation and K-ras mutations in patients at elevated risk for CRC. The subset of patients used in our study is unique since they do not have a personal history of CRC nor an existing cancer, but are considered at elevated risk because of a positive family history and/or a personal history of benign polyps or adenomas. In our study, ACF were identified during screening colonoscopy by in situ surface dye staining and video/biopsy chromendoscopy, affording a much higher level of resolution than traditional colonoscopy, and thus providing a more accurate method for ACF detection (9). For example, in a recent study, it was found that traditional flexible sigmoidoscopy was rate method for ACF detection (9). For example, in a recent study, it was found that traditional flexible sigmoidoscopy was rate method for ACF detection (9). For example, in a recent study, it was found that traditional flexible sigmoidoscopy was rate method for ACF detection (9). For example, in a recent study, it was found that traditional flexible sigmoidoscopy was rate method for ACF detection (9). For example, in a recent study, it was found that traditional flexible sigmoidoscopy was rate method for ACF detection (9). For example, in a recent study, it was found that traditional flexible sigmoidoscopy was rate method for ACF detection (9). For example, in a recent study, it was found that traditional flexible sigmoidoscopy was rate method for ACF detection (9). For example, in a recent study, it was found that traditional flexible sigmoidoscopy was rate method for ACF detection (9). For example, in a recent study, it was found that traditional flexible sigmoidoscopy was rate method for ACF detection (9).
present in 67% (2 out of 3) of ACF with K-ras mutations, but only 20% (4 out of 20) of ACF without K-ras mutations. \( P > 0.05 \). Although the correlation did not achieve statistical significance as a result of only a limited sample size, the observed trend does imply that the two events are not necessarily mutually exclusive. Additional analysis of MSI status, however, would be required to confirm whether this synergy is restricted to MMR-deficient lesions, or is dependent upon other factors, such as lesion histology.

In conclusion, our data provide the first evidence that an important tumor suppressor gene, RASSF1A, has undergone promoter hypermethylation within the ACF of patients undergoing surveillance colonoscopy. Importantly, this epigenetic alteration was identified within the colonic epithelium of normal subjects at elevated risk of CRC, but with no evidence of synchronous colon cancer. A significant consequence of this epigenetic event is transcriptional silencing of RASSF1A, occurring exclusively within the aberrant crypt cells, but not in abutting normal epithelium. These data argue against the possibility of a methylation ‘field effect’, and further suggest that epigenetic silencing may in fact represent one of the earliest alterations in the CRC pathway.

Acknowledgements

We thank the Olympus Corporation for generously providing the endoscopy instrumentation and the Yellin Golf Foundation for their generous support and NIH grant CA81428.

Conflict of Interest Statement: None declared.

References


Received January 13, 2006; revised February 1, 2006; accepted February 6, 2006.