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Advances in Brief

Somatic *APC* and K-*ras* Codon 12 Mutations in Aberrant Crypt Foci from Human Colons[1]

Andrew J. Smith, Hartley S. Stern,[2,3] Mark Penner, Kazy Hay, Angela Mitri, Bharati V. Bapat,[2] and Steven Gallinger[2]

Departments of Surgery [A. J. S., H. S. S., K. H., B. V. B., S. G.] and Pathology [A. M., B. V. B.], and the Samuel Lunenfeld Research Institute [H. S. S., M. P., S. G.], Mount Sinai Hospital, University of Toronto, Toronto M5G 1X5, Canada

Abstract

Aberrant crypt foci (ACF) are microscopic lesions which have been postulated to precede the development of adenomatous polyps, the precursors to colorectal cancer. *APC* and *ras* gene mutations have been shown to be important early molecular events in the development of colorectal neoplasms. The objective of this study was to establish the nature and frequency of these two genetic alterations in ACF harvested from human colorectal resection specimens. One hundred and fifty-four ACF comprised of between 1 and 56 crypts were harvested from the grossly normal mucosa of colorectal resection specimens of 28 patients with varying pathological diagnoses. One hundred and twenty-five ACF from 20 colons were screened for the presence of K-*ras* codon 12 mutations with a polymerase chain reaction/restriction enzyme-based method. The *APC* gene mutation cluster region was screened in 65 ACF from 20 colons using a polymerase chain reaction/single strand conformation polymorphism technique. Putative mutations were confirmed by direct sequencing. K-*ras* codon 12 mutations were identified in 13% (16 of 125) of ACF. We also identified *APC* mutations in 4.6% (3 of 65) of ACF. The results of this study demonstrate that both *APC* and K-*ras* mutations occur in ACF. These observations support the role of the ACF as a colorectal cancer precursor and provide further insight into the early genetic changes which occur during colorectal tumorigenesis.

Introduction

CRC[4] develops through a series of distinct histological stages, encompassing a progression from normal mucosa to premalignant adenomatous polyps to invasive cancer (1). The ACF has been proposed as a distinct histological lesion that precedes the development of adenoma (2–4). This microscopic lesion consists of clusters of abnormally large, darkly staining, slightly elevated mucosal crypts. ACF were initially described in association with a carcinogen-induced model of colorectal tumorigenesis in rodents (5). Epidemiological and histological features of the ACF in rodent models support its role as a microscopic precursor to benign neoplastic polyps and cancers. ACF similar to those identified in rodents have been characterized in human colons (3, 4). Furthermore, recent data suggest that ACF are present at an increased frequency in the colons of patients with CRC compared to patients with benign colonic diseases (3).[5] Thus, the ACF may have an important role as a biomarker predictive of CRC risk.

Mutations of various proto-oncogenes and tumor suppressor genes are known to accumulate during the progression from normal to malignant tissue. *APC* and K-*ras* gene mutations have been shown to be important alterations in the development of CRC (6, 7). *APC* mutations are likely one of the earliest events in CRC, since their presence has been noted in macroscopic adenomas as small as 0.3 cm in diameter (7, 8). To date, however, *APC* mutations have not been identified in human ACF. In contrast, previous studies have demonstrated *ras* mutations in rat (9, 10) and in a small series of human ACF (11). The purpose of the present study was to further strengthen the link between ACF and CRC by characterizing the nature and frequency of K-*ras* and *APC* mutations in ACF.

Materials and Methods

Colectomy Specimens. Twenty-eight colectomy specimens from patients undergoing resection for CRC, FAP, ulcerative colitis, diverticular disease, and rectal prolapse were obtained directly from the operating room. In all cases, grossly normal mucosal strips (mean area, 12 cm^2) were dissected from the underlying submucosa. For CRC cases, colonic mucosa was sampled at a point approximately 10 cm proximal to the tumor. The specimens were stored between filter paper in 10% buffered formalin prior to sampling for ACF and microscopically normal mucosa.

ACF and Microscopically Normal Mucosa Samples. The mucosal strips were stained with a 0.1% solution of methylene blue and examined under a ×30 dissecting microscope (Cambridge Instruments, Buffalo, NY). ACF were identified and microdissected from the surrounding normal epithelium (Fig. 1). Microscopically normal mucosa of comparable size to ACF (<1 mm in diameter) was similarly harvested. Both ACF and normal mucosal samples were then stored at −70°C for subsequent DNA extraction and mutation analysis. The number of crypts per ACF and the total number of ACF per cm^2 were recorded.

Tumor Samples. Samples of colorectal tumors were obtained from 11 of the patients with CRC. Paraffin sections were stained with hematoxylin and eosin and examined histologically. Neoplastic tissue was microdissected, and DNA was extracted as described below.

DNA Extraction. Each sample was suspended in 50–200 μl of a lysis solution containing 10 mM Tris-HCl (pH 8.3), 100 mM KCl, 2.5 mM $MgCl_2$, and 0.45% Tween-20 (Amersham, Oakville, ON) and then overlaid with mineral oil and incubated at 95°C for 10 min. Proteinase K (Boehringer Mannheim, Laval, Québec, Canada) was added to a final concentration of 2.5 mg/ml. Samples were incubated for 1 h at 65°C, followed by a period of 10 min at 95°C to inactivate the Proteinase K. All DNA samples were stored at −20°C.

K-*ras* Codon 12 Mutations. We used a mismatched primer PCR combined with restriction enzyme analysis modified from the method of Levi *et al.* (12). Briefly, DNA extracted from ACF was amplified by PCR in a reaction volume of 100 μl, using the same "A" and "B" primers (200 ng) and the same

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[2] These authors contributed equally to the project.

[3] To whom requests for reprints should be addressed, at Department of Surgery, University of Ottawa, Suite 105, 1053 Carling Ave., Ottawa, Ontario K1Y 4E9, Canada.

[4] The abbreviations used are: CRC, colorectal cancer; ACF, aberrant crypt focus/foci; APC, adenomatous polyposis coli; FAP, familial adenomatous polyposis; PCR, polymerase chain reaction.

[5] A. J. Smith, S. Gallinger, K. Hay, B. V. Bapat, and H. S. Stern. Evidence for increased prevalence of ACF in colons of patients with colorectal cancer, manuscript in preparation.

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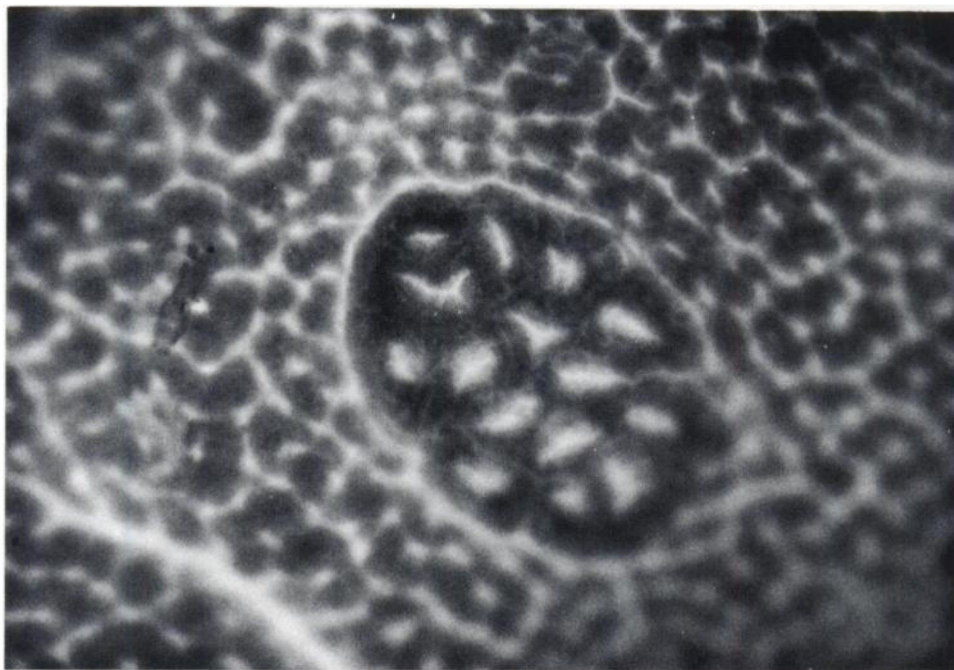

Fig. 1. A single ACF identified within grossly normal mucosa (methylene blue, × 100).

amplification profile (40 cycles) as described by Levi *et al.* (12). Following this amplification, the DNA was digested with *Bst*NI (New England Biolabs, Beverly, MA). Digested DNA was diluted 1:100 with H_2O, and 10 μl were reamplified (40 cycles) in a total volume of 100 μl using the same 5′ "A" primer and a nested 3′ primer (5′ TCCACAAAGTGATTCTGAAT). The second PCR product was then digested with *Bst*NI. Mock-digested and *Bst*NI-digested products were electrophoresed on 12% polyacrylamide gels. Wild-type K-*ras* sequence harbors a *Bst*NI restriction site at codon 11, which is abolished by nucleotide substitutions at the first or second bases of K-*ras* codon 12; thus, samples containing K-*ras* codon 12 mutations failed to cut with *Bst*NI. All mutations detected by the above assay were characterized by directly sequencing the second PCR product after purification in a Microcon 30 microconcentrator (Amicon, Beverly, MA). The 3′ primer was used in sequencing using the CircumVent Thermal Cycle Sequencing Kit (New England Biolabs, Beverly, MA).

Detection of Somatic *APC* Mutations. Screening for somatic *APC* mutations was performed using PCR and single-strand conformation polymorphism analysis as described previously (13). The mutation cluster region of *APC* was amplified using six overlapping primer pairs from *APC* codons 1044 to 1554 (14, 15). Samples were electrophoresed at room temperature on 5% polyacrylamide/10% glycerol gels. The possibility of carry-over PCR product contamination was minimized by confirming single-strand conformation polymorphism-positive results in independent and repeated PCR reactions. All positive conformers were excised from the dried gels, and DNA was eluted with distilled water. Eluted DNA was reamplified and directly sequenced to characterize the mutations, as described above.

Results

DNA samples from a total of 154 ACF harvested from 28 patients were evaluated for K-*ras* codon 12 and/or *APC* mutations; 125 ACF from 20 colons were tested for K-*ras* mutations, while 65 ACF from 20 colons were analyzed for *APC* mutations. Thirty-six ACF were evaluated for both K-*ras* and *APC* mutations. One hundred samples of microscopically normal mucosa and 11 colorectal cancer samples were also studied. Patient and ACF characteristics are shown in Table 1. Table 2 describes the pathological features of the colons from which the ACF were harvested.

K-*ras* codon 12 mutations were detected in 13% (16 of 125) of ACF samples from 10 colons (Table 3). Mutations were GGT→GTT (7 of 16) or GGT→GAT (9 of 16) single base substitutions. A K-*ras* mutation was found in 1% (1 of 100) of normal mucosal samples. This represents a significantly higher rate of K-*ras* mutation in the ACF samples compared to the normal mucosal samples ($P < 0.001$, Student's *t* test). In addition, K-*ras* mutations were found in 45% (5 of 11) of primary tumors evaluated. In some instances, different ACF and/or CRC samples harvested from the same colon resection specimen were found to harbor different K-*ras* codon 12 mutations. Moreover, in some colons, the ACF demonstrated K-*ras* codon 12 mutations, but the corresponding CRC samples did not (Table 3).

APC analysis was performed on 65 ACF from 20 colorectal resection specimens. Pathological diagnoses of resection specimens included CRC (12 colons, 30 ACF), FAP (5 colons, 28 ACF), ulcerative colitis (2 colons, 6 ACF), and diverticulosis (1 colon, 1 ACF). Somatic *APC* mutations were found in 4.6% (3 of 65) of ACF. All three ACF with mutations were from a single FAP patient (DJ); a total of 15 ACF were examined from this patient. All mutations were 5-base pair deletions (ACAAA) at codon 1061, which result in a premature termination of the predicted APC protein at nucleotides 3189–3191.

Discussion

The present study represents the largest series of human ACF examined to date. In this report, we have demonstrated the presence of K-*ras* codon 12 mutations and *APC* mutations in ACF from human colons. Since alterations in these two genes are thought to be important events in colorectal tumorigenesis (16), identification of these mutations in ACF supports the role of the ACF as a CRC precursor.

We concentrated on the detection of K-*ras* codon 12 mutations in human ACF, since mutations at this site constitute the majority of *ras* mutations in colorectal tumors (6, 17). Of 125 ACF from the 20 colons studied, we have demonstrated K-*ras* codon 12 mutations in 13%. This mutation rate is similar to that demonstrated for small, macroscopic adenomas. Moreover, the 45% frequency of *ras* mutations in the tumor samples is in agreement with previous findings (6, 17). The K-*ras* mutations in our ACF series were G→A transitions and G→T transversions in the second position of codon 12. Given that these are also the most common mutations observed in studies of colorectal neoplasms, this finding supports the hypothesis that these

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Table 1 *Patient demographics and ACF samples*

Patient	Age	Diagnosis	No. of ACF assayed	Crypts per ACF[a]
144	62	CRC	5	3–50
145	63	CRC	7	7–50
146	56	CRC	7	2–50
147	59	CRC	2	9–25
148	66	CRC	1	3
149	41	FAP	10	1–50
DJ	35	FAP	29	1–56
155	52	FAP	4	ND[b]
158	55	CRC	1	ND
172	19	CRC	1	3
183	20	FAP	3	5–40
185	73	CRC	1	5–50
190	32	UC[c]	7	3–10
191	30	UC	6	1–50
192	41	FAP	6	2–31
194	68	CRC	4	3–31
195	68	CRC	1	4
196	70	CRC	8	2–50
197	82	CRC	10	1–50
198	40	UC	5	1–25
200	63	Diverticulosis	1	2
202	62	CRC	5	4–13
203	20	UC	5	1–6
208	49	CRC	2	ND
211	43	FAP	13	ND
233	63	CRC	4	1–26
234	48	CRC	6	3–50
236	60	Rectal prolapse	0	ND

[a] Range.
[b] ND, not determined.
[c] ACF from ulcerative colitis (UC) colons were sampled from areas of grossly normal mucosa.

tumors arise from ACF (6, 17). Our results demonstrate that not all ACF in a given individual harbor the same K-*ras* codon 12 mutations, and tumors do not always have the same mutation as the ACF analyzed from the same colon. This is not surprising because each lesion probably arises from an independent clone, and the same mutagenic environmental influences can produce different mutations (18, 19). Further study with larger numbers of ACF may show that one particular mutation confers a greater chance of progression to macroscopic tumor than another.

Pretlow *et al.* (11) have demonstrated K-*ras* codon 12 mutations in 73% (11 of 15) of the ACF examined in their study. These ACF were harvested from the colons of six patients with CRC and one patient with benign colonic disease. The higher mutation frequency observed by Pretlow *et al.* (11) may in part be due to the smaller sample size used (15 ACF) and the types of colons studied. In our series, the ACF derived from patients with CRC had a 25% frequency of K-*ras* mutations, compared to a 4% rate in the ACF harvested from non-CRC colons. Moreover, K-*ras* mutations were found in 5 of 6 (83%) ACF from 1 of the colons in our series. Extensive sampling of such an individual's ACF may significantly increase the observed frequency of mutation in this type of study when the sample size is small.

Table 2 *Pathological features of colons from which mucosal samples were harvested and frequency of K-ras codon 12 mutations*

Specimen diagnosis and colonic pathology	No. of ACF assayed	No. of colons	No. of K-*ras* mutations
ACF from CRC	53	12	13
ACF from FAP	51	3	2
ACF from UC[a]	20	4	1
ACF from diverticulosis	1	1	0
Normal mucosa[b] from CRC	54	6	1
Normal mucosa from diverticulosis and rectal prolapse	28	4	0
Normal mucosa from UC	18	2	0
Adenocarcinoma from CRC	NA[c]	11	5

[a] UC, ulcerative colitis.
[b] Microscopically normal pieces of mucosa similar in size to ACF (<1 mm in diameter).
[c] NA, not applicable.

Mutations of the *APC* gene have not been demonstrated previously in ACF. In this study, we found somatic *APC* mutations in 3 of the 65 ACF evaluated. All three mutations were a 5-base pair deletion (ACAAA) at codons 1061–1063 and were identified in ACF harvested from a single FAP patient. Interestingly, we did not find somatic *APC* mutations in ACF assayed from the colons of 19 other patients. This indicates that mutation of *APC* is a relatively rare event at this early histological stage. This is a significant observation, since previous studies have demonstrated a uniformly high mutation frequency of approximately 60% for both benign colorectal adenomas and carcinomas (7, 15). Furthermore, somatic *APC* mutations have been identified in adenomas as small as 5 mm in diameter. These studies have supported the concept that *APC* mutation is a relatively early event in colorectal tumorigenesis (7). However, our observation of a low mutation frequency in ACF suggests that *APC* mutation is unlikely to function as a significant contributor to the genesis of ACF.

Colorectal neoplasia is believed to be initiated by somatic mutations which result in clonal expansion of a single cell. Additional mutations accumulate in a subset of daughter cells, thus affording these an even greater growth advantage. Further clonal proliferation ultimately results in the development of a macroscopic adenoma or polyp (16, 18, 19). The ACF is hypothesized to represent the first identifiable phenotypic expression of a clonal expansion derived from a single colonic epithelial cell. Current paradigms contend that both *ras* and *APC* mutations are important early alterations in colorectal tumorigenesis (16). However, the implication of our results and the

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Table 3 *Colon specimens from which ACF and/or CRC containing K-ras codon 12 mutations were identified*

Patient	No. of ACF assayed	No. of mutations	Nature of mutation(s)	Mutation in CRC from corresponding colon sample
144	5	1	GAT	GTT
145	7	2	GAT, GTT	wt[a]
146	7	1	GAT	GAT
147	2	0	wt	GAT
148	1	0	wt	GTT
191	6	1	GTT	NA[b]
194	4	1	GTT	wt
196	6	1	GTT	wt
197	8	1	GAT	wt
208	2	1	GAT	GAT
211	13	2	GTT(2)	NA
234	6	5	GAT(4), GTT(1)	wt

[a] wt, wild-type (GGT).
[b] NA, not applicable because the colon resection was for benign disease.

observations of Pretlow *et al.* (11) on K-*ras* mutation frequency is that *ras* activation is more common than *APC* inactivation at the ACF stage. This is consistent with a hypothesis that mutational activation of K-*ras* may be one of the key phenomena in the generation of the ACF phenotype. In contrast, because mutation of *APC* is a less frequent finding in ACF but is commonly observed in macroscopic adenomas, its importance may lie in facilitating progression from ACF to the adenoma stage. Two additional observations are consistent with the theory that an infrequent key alteration is necessary for progression from the ACF stage: (*a*) in a carcinogen-induced rodent model of CRC, we have demonstrated that, although some ACF clearly progress to malignant tumors, the majority of ACF regress;[6] and (*b*) it is known that CRC patients have one to two ACF per cm^2 of normal mucosa (3) and, therefore, many hundreds of ACF throughout their entire colon. However, only 25% of individuals are found to have synchronous adenomatous polyps at the time of colectomy for CRC and only 4% go on to develop a metachronous cancer (1). Both of these findings support the concept that only a small minority of ACF acquire the growth advantage necessary to progress to a macroscopic size.

Further investigations are necessary to define the features of ACF which are most likely to predict progression to macroscopic adenoma and carcinoma. Such studies could eventually allow prediction of CRC risk through assessment of an individual's ACF. K-*ras* and *APC* mutations in ACF may in part denote the potential of a given ACF to progress, but it is likely that other genetic markers will also be informative. In addition to the genetic features of ACF, it is important to note that morphological analysis reveals that a subset of ACF manifest the nuclear and cellular features of dysplasia and, thus, might be considered more likely to progress to more advanced lesions (3). Therefore, future studies might evaluate a battery of variables including ACF frequency, histology, and various molecular markers to predict progression during colorectal neoplasia.

[6] B. Shpitz, K. Hay, A. Medline, W. R. Bruce, S. B. Bull, S. Gallinger, and H. S. Stern. The natural history of aberrant crypt foci: a surgical approach, submitted for publication.

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