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

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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 from 20 colons were screened for the presence of K-ras codon 12 mutations with a polymerase chain reaction/restriction enzyme-based method. 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 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). 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 cm²) 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 cm² 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 MgCl₂, 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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3 The abbreviations used are: CRC, colorectal cancer; ACF, aberrant crypt focus/foci; APC, adenomatous polyposis coli; FAP, familial adenomatous polyposis; PCR, polymerase chain reaction.
amplification profile (40 cycles) as described by Levi et al. (12). Following this amplification, the DNA was digested with BstNI (New England Biolabs, Beverly, MA). Digested DNA was diluted 1:100 with H2O, 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' TCCACAAAAGTGAATTCCTGAAT). The second PCR product was then digested with BstNI. Mock-digested and BstNI-digested products were electrophoresed on 12% polyacrylamide gels. Wild-type K-ras sequence harbors a BstN1 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 BstNI. 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
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.

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
future studies might evaluate a battery of variables includ
entire colon. However, only 25% of individuals are found to have
cancer. Such studies could eventually allow prediction of
size.
manifest the nuclear and cellular features of dysplasia and, thus, might
informative. In addition to the genetic features of ACF, it is important
characterization of stereomicroscopically identified preneoplastic lesions during dimethyl-

observations of Pretlow et al. (11) on K-ras mutation frequency is that
activation is more common than APC inactivation at the ACF
dominant phenotype in the generation of the ACF
stage. This is consistent with a hypothesis that mutational activation of K-ras
cancer. In contrast, because mutation of APC is a less frequent
important role in facilitating progression from ACF to the
daemonia 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 pro-
gress to malignant tumors, the majority of ACF regress; and (b) it is
known that CRC patients have one to two ACF per cm² of normal
mucosa (3) and, therefore, many hundreds of ACF throughout their
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 includ-
ing ACF frequency, histology, and various molecular markers to
predict progression during colorectal neoplasia.

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### References

3. Roncucci, L., Medline, A., and Bruce, W. R. Classification of aberrant crypt foci and
microadenomas in human colon. Cancer Epidemiol. Biomarkers Prev., 1: 57—60,
4. Pretlow, T. P., Barrow, B. J., Ashton, W. S., O'Riordan, M. A., Pretlow, T. G.,
Jurczek, J. A., and Stellato, T. A. Aberrant crypts: putative preneoplastic foci in
5. Sandfor, P., Heinzel, S., Falzer, P., Gunschmidt, S., and Riecken, E. O. Charac-
terization of stereomicroscopically identified preneoplastic lesions during dimethyl-
M., Nakamura, Y., White, R., Smits, A. M. M., and Bos, J. L. Genetic alterations
Thibodeau, S. N., Vogelstein, B., and Knizler, K. W. APC mutations occur early
Inactivation of both APC alleles in an early stage of colon adenomas in a patient with
9. Shivasvark, N., Tang, Z., Ferreira, A., Nasim, S., Garett, C., and Alabaster,
Joseph. Sequential analysis of K-ras mutations in aberrant crypt foci and colorectal tumors
induced by azoxymethane in Fischer-344 rats on high-risk diet. Carcinogenesis (Lond.),
H. S., and Gallinger, S. K-ras mutations in aberrant crypt foci, adenomas and
adenocarcinomas during azoxymethane-induced colon cancer. Carcinogene-
sis (Lond.), 14: 1777—1781, 1993.
Marshall, C. J. Multiple K-ras codon 12 mutations in cholangiocarcinomas demon-
strated with a sensitive polymerase chain reaction technique. Cancer Res., 31:
somatic APC gene mutations in perianampullary adenomas in a patient with familial
14. Miyoshi, Y., Nagee, H., Hori, S., Ishii, K., Nakatsu, A., Aoki, T., Miiki,
Y., Mori, T., and Nakamura, Y. Somatic mutations of the APC gene in colorectal
mutations of the adenomatous polyposis coli gene in colorectal tumors. Cancer Res.,
17. Laurent-Puig, P., Olschow, S., Delattre O., Valid, P., Meot, T., Mosseri, V.,
Salmon, R. J., and Thomas, G. Association of K-ras mutation with differentiation
and tumor-formation pathways in colorectal carcinoma. Int. J. Cancer, 49: 220—223,
18. Ponder, B. A. J., and Wilkinson, M. M. Direct examination of the clonality of
carcinogen-induced colorectal epithelial dysplasia in chimeric mice. J. Natl. Cancer

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

<table>
<thead>
<tr>
<th>Patient</th>
<th>No. of ACF assayed</th>
<th>No. of mutations</th>
<th>Nature of mutation(s)</th>
<th>Mutation in CRC from corresponding colon sample</th>
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<tr>
<td>144</td>
<td>5</td>
<td>1</td>
<td>GAT</td>
<td>GTT</td>
</tr>
<tr>
<td>145</td>
<td>7</td>
<td>2</td>
<td>GAT, GTT</td>
<td>wt</td>
</tr>
<tr>
<td>146</td>
<td>7</td>
<td>1</td>
<td>GAT</td>
<td>GTT</td>
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<td>2</td>
<td>0</td>
<td>wt</td>
<td>GAT</td>
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<tr>
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<td>0</td>
<td>wt</td>
<td>GAT</td>
</tr>
<tr>
<td>191</td>
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<td>1</td>
<td>GTT</td>
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</tr>
<tr>
<td>234</td>
<td>6</td>
<td>5</td>
<td>GAT(4), GTT(1)</td>
<td>wt†</td>
</tr>
</tbody>
</table>

† wt, wild-type (GOT).
‡ NA, not applicable because the colon resection was for benign disease.
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