

## Emergence of Adenomatous Aberrant Crypt Foci (ACF) from Hyperplastic ACF with Concomitant Increase in Cell Proliferation<sup>1</sup>

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### Abstract

To investigate the relationship between aberrant crypt foci (ACF) and colon neoplasia in colorectal carcinogenesis, we evaluated 433 ACF, which were collected from the grossly normal mucosa of surgical specimens from 57 patients with colorectal cancer. The ACF ranged in size from 3 to 412 aberrant crypts/focus. Large ACF ( $\geq 50$  crypts/focus) comprised 25% of the total ACF studied. Histopathologically, 65% (67 of 103) of large ACF were diagnosed as hyperplasia, 10% (10 of 103) as adenoma, and 1% (1 of 103) as within normal colorectal mucosa. The remaining 24% (25 of 103) were diagnosed as "stage I abnormality crypts," which were characterized by their extension of the proliferative compartment to the surface of crypts but with no changes in the major site of proliferation, as designated by E. E. Deschner [Pathol. Annu., 18 (Part 1): 205–219, 1983]. Of the 25 stage I abnormality ACF, 7 ACF coexisted with hyperplasia. Of 10 adenomatous ACF, two coexisted with stage I abnormality crypts. A *K-ras* codon 12 mutation was identified in 85% (93 of 109) of large ACF. The proliferative activity of stage I crypts was significantly higher than that of hyperplastic crypts in the same ACF. These observations suggest that some hyperplastic ACF may develop into adenomatous ACF by way of stage I abnormality ACF with concomitant acquisition of higher proliferative activity through some genetic and/or epigenetic changes.

### Introduction

ACF<sup>3</sup> were first identified microscopically in methylene blue-stained, whole-mount preparations of colonic mucosa from carcinogen-treated rodents (1–4). Aberrant crypts are distinguished from normal crypts in these preparations by their asteroid- or oval-shaped lumen and elevated thick epithelium. Pathological features of ACF found during carcinogenesis in these rodents seem to be heterogeneous, from mildly atypical to dysplastic crypts. ACF have also been found in the colons of patients both with and without colorectal cancer (5–7). A high frequency of *K-ras* mutation and a low frequency of *APC* mutation have been found in ACF (8–12). In humans, ACF show increased cell proliferation and heterogeneity in their pathological appearance (5–7, 13), as was found in rodents (3, 14). From the above evidence, ACF are widely accepted as being putative neoplastic lesions; however, their histological characteristics have yet to be determined definitively. Yamashita *et al.* (8, 9) reported that characteristics of human ACF were the apical branching of aberrant crypts and harbored a high frequency of *K-ras* mutation. Their main histological finding was hyperplasia of glandular epithelium in human ACF with less than 49 crypts/focus ( $<0.5$  mm in diameter). The high

incidence of *K-ras* mutation in these ACF is a unique feature, in contrast to the low incidence of *K-ras* mutation found in small ( $<5$  mm in diameter) adenomatous polyps (15). The almost exclusive appearance of hyperplasia in these ACF indicates that they are not related to neoplasia on the basis of morphology, although they were supposed to be a clonal expansion of cells harboring the *K-ras* mutation. To determine whether ACF develop into more aggressive neoplasia, we evaluated the histological features and genetic and biochemical characteristics of a large number of ACF with more than 50 crypts/focus in Japanese patients with colorectal cancer.

### Materials and Methods

**Colorectal Specimens.** Human colorectal specimens were obtained from 57 patients (58 samples) who underwent surgery for sporadic colorectal cancers at the National Cancer Center Hospital East from August 1994 to March 1995. These patients, ages 38–82 years, had no history of familial adenomatous polyposis, ulcerative colitis, or hereditary nonpolypoid colorectal cancer. Approximately 36-cm<sup>2</sup> macroscopically normal mucosa were removed from each colon and then fixed with 10% buffered formalin for about 12 h.

**ACF Samples.** All mucosal strips were examined under a stereo microscope after staining with a 0.1% solution of methylene blue. Aberrant crypts were distinguished from normal crypts by their deeper blue color, larger size, the oval shape of the luminal opening, and the slight mucosal elevation (6). All of the large ACF ( $\geq 50$  crypts/focus) and 40 of the small ACF ( $\leq 49$  crypts/focus) were divided into two pieces. One was used for blind pathological analysis by one of the authors (T. H.). The other piece of ACF sample was used for DNA extraction after removal of as much normal mucosa as possible.

**Histological Criteria.** Histologically, ACF were classified into four groups: normal mucosa, hyperplasia, "stage I abnormality crypt," and adenoma. Stage I abnormality crypt was first designated by Deschner (16). It showed extension of proliferative compartment to the surface of the crypt but with no change in the major site of proliferation, whereas stage II abnormality exhibits a shift in the major zone of cell proliferation to the middle and upper portion of the gland, as in adenoma. Therefore, stage I abnormality crypts had been thought to be a precursor lesion of adenoma (16). The hyperproliferation of stage I abnormality was diagnosed by hematoxylin and eosin and PCNA stainings in this study. A reduction in the number of goblet cells, branching of crypts, and/or stratification of nuclei were found in most of stage I abnormality crypts. These characteristics were exactly found in oligocryptal adenoma that were seen in the normal-appearing mucosa of patients with familial polyposis (17).

**DNA Extraction.** The genomic DNA was extracted from ACF by serial digestions with proteinase K and RNase A and then concentrated with a Microcon model 100 (Amicon, Beverly, MA; Refs. 18 and 19). An aliquot of 1  $\mu$ l DNA solution was used as a template for PCR.

**Analysis of *K-ras* Codon 12 Mutations.** Mutations in the *K-ras* gene codon 12 were initially screened by mismatched, primer-mediated PCR amplification for 40 cycles, followed by RFLP analysis using a restriction endonuclease *Mva*I (Takara, Kyoto, Japan; PCR-RFLP). The sequences of the set of primers and conditions for PCR targeting codon 12 were as described previously (20). PCR products encoding the wild-type and mutant sequences were distinguished as 114- and 143-bp fragments, respectively, by digestion with the restriction enzyme *Mva*I. Mutations in *K-ras* codon 12 are detectable

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<sup>3</sup> The abbreviations used are: ACF, aberrant crypt foci; PCNA, proliferating cell nuclear antigen; APC, adenomatous polyposis coli.

by this method when mutated DNA comprise more than 10% of genomic DNA.

**Immunohistochemistry.** To assess the levels of PCNA in the nuclei of the epithelial cells, deparaffinized sections were heated in a hot water bath at 90°C for 10 min in 0.01 M PBS (pH 7.2) and then stained by the avidin-biotin-peroxidase method using a 1:10,000 dilution of the mAb PC10 (DAKO Corp., Carpinteria, CA). Vertical sections of aberrant and normal crypts were used for determination of the PCNA-labeling index.

**Statistical Analyses.** Statistical analysis of data was performed using the Bonferroni/Dun test with Stat View for the Macintosh, version 4.0 software (Abacus Concepts, Berkeley, CA).  $P < 0.05$  was considered statistically significant.

## Results

ACF were identified in 50 colorectal specimens from 57 patients with colorectal cancer. Data for all 433 foci are summarized in Table 1. The incidence of ACF was significantly higher in the rectum than in the ascending and transverse colons ( $P < 0.05$ ). The incidence of ACF was also significantly higher in the sigmoid colon than in the transverse colon ( $P < 0.05$ ; Table 1). These findings are consistent with results in previous reports (6–10). The number of ACF/area was similar in males and females,  $0.27 \pm 0.32/\text{cm}^2$  and  $0.28 \pm 0.33/\text{cm}^2$ , respectively. There was no difference in crypt multiplicity between specimens from different locations of the colon and rectum. Large ACF, with more than 50 crypts/focus, accounted for 25% (109 of 433) of total ACF.

Because the main goal of the present study was to know what lesions ACF may progress to, the surface morphology and histopathology of the large ACF were examined carefully. The histopathological examination revealed that 65% (67 of 103) of large ACF consisted of hyperplasia of the epithelium; 10% (10 of 103) were adenomas; and 1% (1 of 103) was classified histopathologically as normal colorectal mucosa. The remaining 24% (25 of 103) consisted of stage I abnormality crypts, which involved extension of the proliferative compartment to the surface of the crypt but with no change in the major site of proliferation. A typical example of ACF consisting of both hyperplasia and stage I abnormality crypts is shown in Fig. 1. The peripheral portion of the focus shows an asteroid pit pattern, which is considered one of the typical surface features of hyperplastic lesions. However, the central portion of the ACF shows a completely different pit pattern, with the long and slender shaped pits that are typical of adenomatous lesions (21). Histologically, this lesion shows a combination of hyperplasia at the periphery and stage I abnormality crypts in the center (Fig. 1B). In stage I abnormality crypts, incomplete disappearance of goblet cells, some elongated nuclei, and/or nuclear stratification were found in addition to expansion of the proliferating area. Those characteristics are common to those of adenomas.

To test possibilities of transition from hyperplastic ACF to stage I abnormality ACF, and stage I abnormality ACF to adenomatous ACF, coexistence of two types of crypts was surveyed carefully. Seven of

25 stage I abnormality ACF coexisted with hyperplastic crypts, and two adenomatous ACF coexisted with stage I abnormality crypts. The proportion of hyperplastic ACF was almost constant in various sizes of ACF, whereas that of adenomatous ACF increased with the size of ACF (Fig. 2). The proportion of stage I abnormality ACF increased with ACF size up to 99 crypts/focus, but the proportion was very low in ACF with more than 150 crypts/focus (Fig. 2). The average crypt multiplicity in adenomatous ACF ( $221.9 \pm 126.9/\text{focus}$ ) was significantly higher than that of hyperplastic ACF ( $97.4 \pm 55.7/\text{focus}$ ) or stage I abnormality ACF ( $72.4 \pm 26.9/\text{focus}$ ;  $P < 0.05$ ).

To clarify any biochemical and/or genetic change(s) that could be responsible for the transition from hyperplasia to adenoma, we examined *K-ras* codon 12 mutations. A *K-ras* mutation in codon 12 was detected in 85% (93 of 109) of the large ACF; however, within this group, the larger the ACF was, the lower were the *K-ras* codon 12 mutations detected (Fig. 3). The rate of *K-ras* activation in ACF with more than 150 crypts/focus was 58% (11 of 19). Of these 19 very large lesions, *K-ras* mutation in adenomatous ACF was 29% (2 of 7); that in hyperplastic ACF was 81% (9 of 11); and that in stage I abnormality ACF was 0% (0 of 1).

We also examined p53 protein nuclear accumulation in large ACF. However, p53 nuclear accumulation was not detected immunohistochemically in any of the ACF (data not shown), which could explain the hyperplasia-adenoma transition.

Cell proliferation activity was examined immunohistochemically by PCNA-positive cells. The distribution of PCNA-positive cells in hyperplastic and adenomatous crypts of ACF was quite different, and that in hyperplastic and stage I abnormality crypts was also quite different. There was no remarkable difference between stage I abnormality and adenomatous crypts. PCNA-positive cells were located from the bottom to the top of adenomatous and stage I abnormality crypts, from the bottom to the middle of hyperplastic crypts (Fig. 1C), and only at the bottom of normal crypts. In ACF consisting of hyperplastic and stage I abnormality crypts, the labeling index of PCNA in stage I abnormality crypts ( $59.4 \pm 3.5\%$ ) was significantly higher than that in hyperplastic crypts ( $40.4 \pm 9.6\%$ ). This increase in the labeling index, therefore, suggests acquisition of neoplastic features in the transition of aberrant crypts from hyperplastic to adenomatous lesions.

## Discussion

Definitive histological classification of ACF is still under debate. In our study and other studies, ACF have been considered to be neoplastic due to the high incidence of *K-ras* mutation (8, 9, 12). Previous studies have shown the independent existence of either hyperplastic or adenomatous ACF (6–8). However, this study clearly demonstrates that a substantial number of ACF contain both hyperplastic and stage I abnormality crypts. Stage I abnormality crypts in ACF were characterized by their hyperproliferation in entire cryptic cells but with no changes in the major site of proliferation and a decrease in goblet

Table 1 Distribution of ACF in the colons of patients with colorectal cancer

Location	No. of patients			Area <sup>a</sup> (cm <sup>2</sup> )	No. of ACF <sup>a</sup> (per cm <sup>2</sup> )	Multiplicity <sup>a,b</sup>
	Total	Male	Female			
Cecum	2	2	0	34 ± 10	0.14 ± 0.12	48 ± 54
Ascending	5	2	3	41 ± 9	0.06 ± 0.08 <sup>c</sup>	78 ± 130
Transverse	7	4	3	38 ± 11	0.03 ± 0.04 <sup>c,d</sup>	68 ± 106
Descending	2	2	0	23 ± 11	0.03 ± 0.04	8 ± 11
Sigmoid	26	14	12	38 ± 22	0.33 ± 0.31 <sup>d</sup>	34 ± 24
Rectum	16	7	9	32 ± 13	0.39 ± 0.39 <sup>c</sup>	32 ± 20

<sup>a</sup> Numbers in each column are the means ± SD.

<sup>b</sup> Number of aberrant crypts/focus.

<sup>c,d</sup> Values with the same superscript are significantly different from each other ( $P < 0.05$ ).



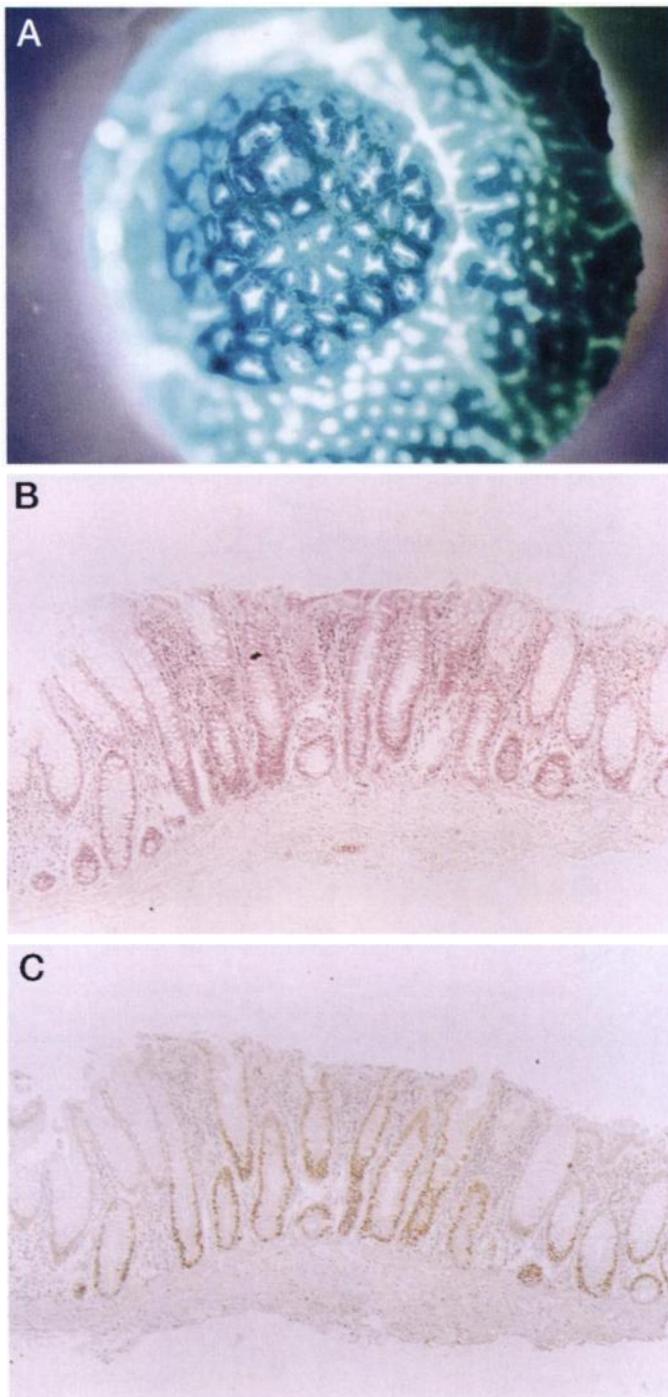


Fig. 1. ACF identified within grossly normal mucosa from a patient with colorectal cancer. A, stereoscopic feature of ACF after staining with methylene blue. The oval or long-shaped pits of aberrant crypts are visible in the center of the ACF, whereas the asteroid-shaped pits are visible in the peripheral portion ( $\times 40$ ). B, histological features of ACF. A section of the same ACF, stained with hematoxylin and eosin ( $\times 80$ ). This ACF is a typical example of ACF with coexisting hyperplastic and stage I abnormality crypts. C, representative cross-section of an ACF immunostained for PCNA. A serial section of the ACF shown was stained with a mAb (PC10) against PCNA, as described in the text ( $\times 80$ ). The central portion of the ACF shows stage I abnormality crypts coexisting with hyperplastic crypts on both sides.

cells. Nuclear stratifications, elongated nuclei, and/or branching of crypts, which are typically seen in adenomatous crypts, are also frequently found in these crypts. As the size of ACF increased, the ratio of stage I abnormality to adenomatous ACF was found to decrease (Fig. 2); it is conceivable that stage I abnormality crypts grow to adenomatous crypts. In fact, 2 of 10 adenomatous ACF

coexisted with stage I abnormality crypts at their periphery. Thus, these findings suggest the existence of a transition stage from hyperplastic to adenomatous ACF. The transition stage was also suggested by the increased number of PCNA-positive cells in stage I abnormality crypts, when compared with the number in hyperplastic crypts in the same ACF. The difference in the PCNA-labeling index between hyperplastic and stage I abnormality crypts in the same ACF further indicates the involvement of genetic and/or epigenetic change(s) in the proposed transition stage.

This idea contradicts the classic histopathological classification to separate hyperplastic and adenomatous regions in the colon. However, the present study does not rule out the widely accepted possibility that some adenomatous lesions occur *de novo*, because small numbers of adenomatous ACF were found in all sizes of ACF.

Of the 433 ACF collected, 25% (109 of 433) were larger than 50 crypts/focus; 8% were larger than 100 crypts/focus; and only 1% were easily visible by the naked eye ( $\geq 300$  crypts/focus). This indicates that only a limited number of ACF grew to large ACF. Because of technical difficulty, we did not count ACF consisting of one or two crypts in this work. Therefore, it is reasonable to speculate that very limited numbers of ACF grow into large ACF. These findings agree well with animal models of carcinogenesis, where many ACF remained small (14).

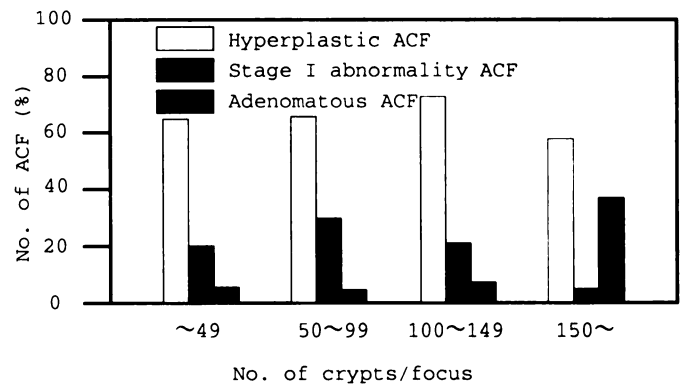


Fig. 2. Histological classification of ACF in relation to their size. ACF, obtained from the colons of patients, were classified into four groups by the number of crypts/focus. Each group of ACF was histologically classified into three types: hyperplasia, stage I abnormality crypt, and adenoma in all the ACF with more than 50 crypts/focus; however, in the case of small ACF ( $\leq 49$  crypts/focus), 40 cases were randomly selected from a total of 324 ACF.

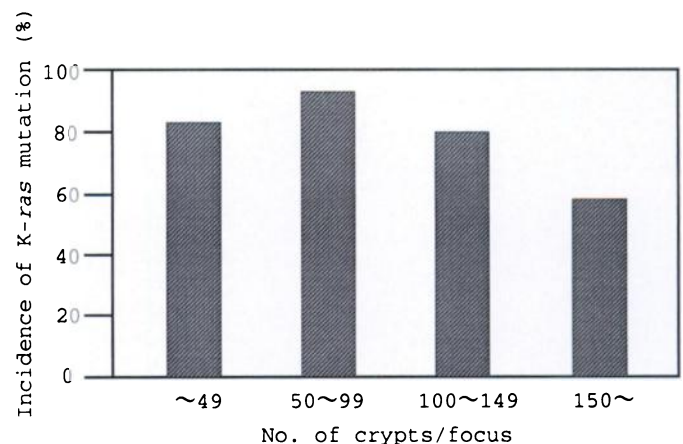


Fig. 3. The frequency of the K-ras (codon 12) mutation in different sizes of ACF. Genomic DNA was isolated from all large ACF and 40 ACF, which were randomly selected from the remaining group ( $< 49$  crypts/focus). PCR-RFLP analysis of the K-ras codon 12 mutation was performed as described in "Materials and Methods."

We tried to elucidate the molecular mechanism underlying the transition from hyperplastic crypt to stage I abnormality and/or adenomatous crypt. Investigation of the *p53* gene did not reveal any evidence to suggest that an alteration of this gene is involved in the above changes, which agrees with previous results obtained by Yamashita *et al.* (8, 9). Therefore, at present, we are examining *APC* gene mutation in ACF to try and determine whether the *APC* gene may play an important role in the progression of ACF from hyperplasia to adenoma. It is widely accepted that the incidence of *K-ras* mutation in small adenomatous polyps is very low. The incidence of *K-ras* mutation only exceeds 10% after adenomatous polyps have grown larger than 10 mm in diameter (15). We and others have also found a very low frequency of *K-ras* mutation in flat-type adenomas and adenocarcinomas (18, 22). In contrast, the present study shows that the incidence of *K-ras* mutation is much higher in ACF. We observed that the larger the size of the ACF, the lower the incidence of *K-ras* mutation. This finding was concomitant with the increase in the proportion of adenomatous ACF. Therefore, there is a possibility that ACF might consist of two distinct lesions, one a “*de novo* adenoma” and the other a *K-ras*-dependent lesion. However, there are two other possible explanations for the above discrepancy. The first possible explanation is that ACF might be a precursor of adenomas having *K-ras* mutations, because a small but distinct proportion of small adenomatous polyps, flat-type adenomas, and adenocarcinomas harbor *K-ras* mutations (15, 17, 22). If this is the case, the adenoma-carcinoma sequence might be divided into *K-ras*-dependent and -independent pathways. The second possible explanation is that the activated *K-ras* gene locus is deleted during the progression of ACF to advanced colorectal cancer through apoptosis, because activated *K-ras* may prevent the enlargement of ACF. Many studies on the loss of heterozygosity in cancer have been performed. The *K-ras* gene is mapped on the short arm of chromosome 12. Although a frequency of loss of heterozygosity of 12p is low, it has been reported in colorectal cancer (23).

The present results strongly suggested that there may be a transition from hyperplastic ACF to adenomatous ACF by way of stage I abnormality ACF with a concomitant increase in cell proliferation; however, further studies are obviously required to confirm this and to determine the mechanism. The present results suggest an important role for ACF and hyperplasia in colorectal carcinogenesis. It should be also emphasized that the time has come to reconsider the term ACF.

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