

Molecular Determinants of Dysplasia in Colorectal Lesions¹Jin Jen,² Steve M. Powell,² Nickolas Papadopoulos, Kelly J. Smith, Stanley R. Hamilton, Bert Vogelstein, and Kenneth W. Kinzler³*The Johns Hopkins Oncology Center, Baltimore, Maryland 21231 [J. J., S. M. P., N. P., K. J. S., B. V., K. W. K.], and Department of Pathology, The Johns Hopkins University, School of Medicine, Baltimore, Maryland 21205-2196 [S. R. H.]*

Abstract

One hallmark of malignant potential is dysplasia, the disruption of normal morphology. While it is generally recognized that cancer is the result of a series of genetic changes, the relationship of these alterations and their timing to the advent of dysplasia remains obscure. To address this issue, 54 small benign colorectal lesions of various malignant potential were analyzed for APC and K-RAS mutations, two alterations which have been implicated in the early stages of colorectal tumorigenesis. APC mutations were closely associated with dysplasia. In contrast, K-RAS mutations were found to be remarkably common in small nondysplastic lesions which apparently have a limited potential to progress to larger tumors. These results provide evidence that the nature and order of genetic changes can have a specific impact on both tumor morphology (e.g., dysplasia) and the likelihood of tumor progression.

Introduction

Colorectal tumors progress through a well recognized series of clinical and histopathological stages ranging from small benign lesions to carcinomas. Early, premalignant colorectal lesions can be readily classified by their morphology and size. Visible tumors (*i.e.*, polyps) are either dysplastic (adenomatous polyps) or nondysplastic (hyperplastic polyps; Ref. 1). Although both types of polyps have a similar frequency and often appear identical at the macroscopic level, dysplastic polyps are distinguished by disruption of the colonic epithelial architecture and cytology and have a well documented tendency to progress to malignancy (2). In contrast, nondysplastic polyps are thought to rarely progress (3). ACF⁴ are microscopic epithelial lesions thought to represent precursors to visible tumors (4, 5). Like their larger counterparts, ACF can be either dysplastic or nondysplastic, with the latter far outnumbering the former (6, 7). Polyps and ACF are remarkably common in humans, and the majority of individuals in developed countries are expected to develop at least one of these lesions over a 70-year lifetime (4, 5, 8). We have attempted to determine whether mutations of specific genes distinguish these various lesions and thereby provide additional insights into their development.

Materials and Methods

Colorectal Lesions. The specimens analyzed were nonmalignant lesions identified in surgical resections from patients with spontaneous colorectal carcinomas. Template DNA was prepared by cryostat sectioning of 34 microdissected polyps as described (9). In each case, twenty 12- μ m sections were

collected for the preparation of DNA and one section immediately before and after the twenty were stained with hematoxylin and eosin. Prior to genetic analysis, the stained sections were examined to determine the fraction of hyperplastic or dysplastic cells. Normal control DNA was prepared in a similar manner from uninvolved mucosa. ACF were identified by staining with methylene blue and tattooed as described (10). The affected colonic mucosa was then carefully excised, cryostat sectioned, and used for DNA preparation.

IVSP Assay. Codons 686-1693 of the APC gene were analyzed for mutations using the IVSP assay as described (11, 12). In brief, two overlapping segments of the APC gene (segments 2 and 3) covering codons 686-1217 and 1099-1693 were amplified using PCR as follows: 100 ng of genomic DNA; 350 ng each of the appropriate primers (Segment 2, 5'-GGATCCTAATAC-GACTCACTATAGGGAGACCACCATGGATGCATGTGGAACTTTGTG-G-3' and 5'-GAGGATCCATTAGATGAAGGTGTGGACG-3'; Segment 3, 5'-GGATCCTAATACGACTCACTATAGGGAGACCACCATGGTTTCTC-CATACAGGTCACGG-3' and 5'-GGAGGATCCTGTAGGAATGGTATC-TCCG-3'); 0.5 μ g single stranded DNA-binding protein (United States Biochemical); and 5.0 units of Taq polymerase were used in a 50- μ l PCR reaction (10 mM Tris-HCl, pH 8.3-50 mM KCl-1.5 mM MgCl₂). Amplifications were performed for 35 cycles of 30-s denaturation (95°C), 90-s annealing (segment 2, 60°C; segment 3, 65°C), and 90-s extension (70°C). All PCR reactions included a 5-min extension period (70°C) after the thirty-fifth cycle. PCR reactions (3 μ l) were used directly without purification as templates in 25 μ l coupled transcription and translation reactions (Promega) containing 40 μ Ci of [³⁵S]methionine translabel (ICN Biochemicals) for 1 h at 30°C. Samples were diluted in sample buffer, boiled for 5 min, and one-tenth was analyzed on 10-20% gradient sodium dodecyl sulfate-polyacrylamide gel. Proteins were visualized by fluorography after impregnating the gel with ENHANCE (DuPont New England Nuclear).

Sequence Analysis. PCR products for sequencing analysis were extracted with phenol-chloroform and precipitated with isopropanol/sodium perchlorate as described (13). PCR templates were sequenced using internal primers and Sequitherm (Epicentre Technologies) following the manufacturer's protocol with the addition of a terminal deoxytransferase extension step at the end of the chain termination reaction (14).

K-Ras Mutation Ligation Assay. All possible alterations at K-RAS codon positions 12a, 12b, and 13b were determined using a modified allele-specific ligation assay (15). The first exon of K-RAS was amplified as described (16) and used as the template for three separate ligation assays (12a, 12b, and 13b). For each ligation assay, 50 ng of PCR product was mixed with 2 ng each of

Table 1 Characteristics of colorectal lesions examined

Lesion type	No. of samples	Average diameter (mm) ^a	No. of affected crypts	No. with APC mutations (%) ^b	No. with K-RAS mutations (%) ^b
Polyps					
Non dysplastic (hyperplastic)	22	5.7 \pm 3.0	>100	0 (0)	5 (22)
Dysplastic	12	4.3 \pm 1.1	>100	10 (82)	3 (25)
ACF					
Non dysplastic	19	<0.5	25.4 \pm 15.3	0 (0)	19 (100)
Dysplastic	1	<0.5	28	1 (100)	0 (0)

^a The average diameter calculation did not include two flat polyps, one dysplastic (14 mm) and one nondysplastic (10 mm).

^b APC and K-RAS mutations were ascertained as described in "Materials and Methods."

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² These authors contributed equally to this work.

³ To whom correspondence should be addressed at The Johns Hopkins Oncology Center, 424 North Bond Street, Baltimore, MD 21231.

⁴ The abbreviations used are: ACF, aberrant crypt foci; APC, adenomatous polyposis coli; IVSP, *in vitro* synthesized protein; PCR, polymerase chain reaction.

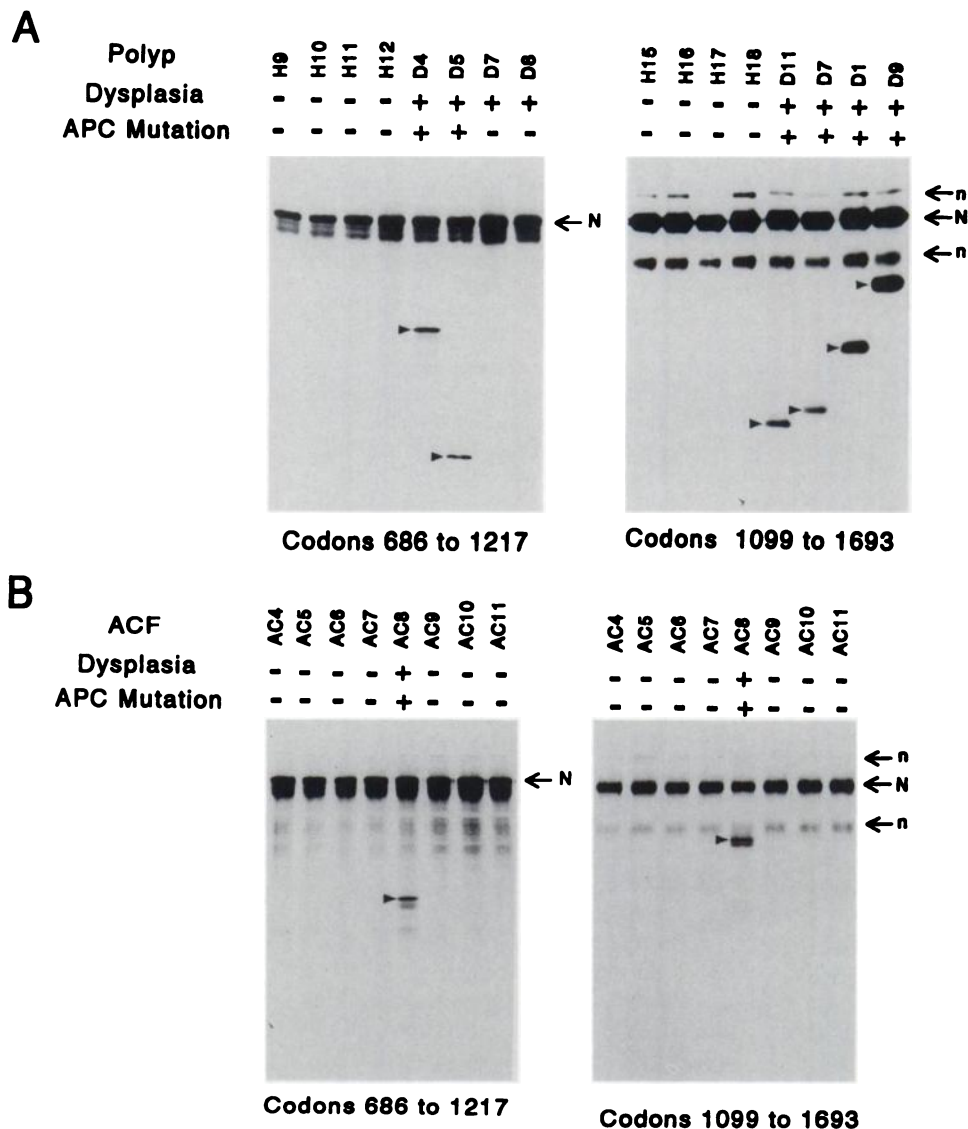


Fig. 1. Analysis of APC mutations in dysplastic and nondysplastic colorectal lesions. Two overlapping segments of APC were analyzed in 34 polyps (A) and 20 ACF (B) using the IVSP assay. Eight representative samples of each are shown. +, presence of dysplasia or an APC mutation; N, band corresponding to normal full length products; n, normally occurring bands corresponding to minor translation products; arrowheads, bands corresponding to truncating mutations.

three mutation specific oligomers, 100 ng blocking oligomer and 2 ng of a common ³²P-labeled oligomer in a 20-μl reaction containing 150 mM NaCl, 10 mM MgCl₂, 100 mM Tris-HCl (pH 7.5), 1 mM spermidine, 1 mM dithiothreitol, 1 mM ATP, 3 μg single strand DNA binding protein (United States Biochemicals). This mixture was heated at 95°C for 5 min and allowed to cool at room temperature for 15 min, at which time 1 unit of T4 ligase was added. The ligation was carried out at 37°C for 1 h and terminated by heat inactivation at

68°C for 10 min. The ³²P-phosphate on the unligated 3' oligomer was removed by the addition of 1 unit alkaline phosphatase and subsequent incubation at 37°C for 30 min. The ligation products were separated on 12% denaturing polyacrylamide gel. The presence and the nature of mutations were determined based on the migration of ligation products formed in control experiments using templates with known K-RAS mutations.

Oligonucleotides used for ligation assays are as follows. Codon 12a: 12a-

Table 2 APC mutations in dysplastic colorectal lesions

Lesion	Size (mm)	Dysplastic cells (%)	IVSP assay ^a	Codon	Mutation
Polyps					
D1	3	70	S3	1420	AGC CCCAGT → AGC CAG _b
D3	5	10	S2	900-990	
D4	5	60	S2	1020	GAA → TAA
D5	6	70	S2	876	CGA → TGA
D6	4	10	S3	1578	TGT → TTGT
D7	4	80	S3	1328	TCA CAG CAC → TCA GCA
D9	4	80	S3	1490	CAT TTT GCC → CAT TTG
D10	5	55	S3	1344	TCA → TAA
D11	4	85	S3	1312	GGA → TGA
D12	14 ^c	<5	S3	1340-1420	

^a APC mutations were initially identified with the use of IVSP assay as described in "Materials and Methods."

^b The mutation could not be identified for technical reasons (see text), and the appropriate location is based on the migration of the abnormal protein observed in the IVSP assays.

^c Flat polyp.

Ser 5'-TTGGAGCTA-3', 12a-Cys 5'-GTTGGAGCTT-3', 12a-Arg 5'-AGTTGGAGCTC-3', and 12a-Common 5'-GTGGCGTAG-3'; codon 12b: 12b-Ala 5'-TGAGCTGC-3'; 12b-Asp 5'-TTGGAGCTGA-3', 12b-Val 5'-GTTGAGCTGT-3, and 12b-common 5'-TGGCGTAGG-3'; codon 13b: 13b-Asp 5'-AGCTGGTGA-3', 13b-Ala 5'-GAGCTGGTGC-3', 13b-Val 5'-GGAGCTGGTGT-3', and 13b-common 5'-CGTAGGCAA-3'. Two 11-mers spanning the ligation junction were used as blocking oligomers to reduce background ligation; these were 5'-AGCTGGTGGCG-3' (12N) and 5'-TGGTGGCGTAG-3' (13N) for codons 12 and 13, respectively.

Results

Because APC (17, 18) and K-RAS (9, 19-23) gene mutations are the earliest mutations observed during colorectal tumorigenesis, we examined these genes in small dysplastic and nondysplastic polyps. We first searched for truncating APC mutations between codons 686 and 1693 using a recently developed IVSP assay (11, 12). This assay detects two-thirds of the APC mutations previously identified in colorectal tumors (11, 17, 18). APC mutations were identified in 10 of 12 (83%) dysplastic polyps but in none of the 22 nondysplastic polyps (Table 1; Examples in Fig. 1A). Eight of the cases with APC mutations were confirmed by nucleotide sequence analysis, which showed that four of the mutations were single base pair changes resulting in nonsense codons and four were small insertions or deletions producing frameshifts (Table 2). The low fraction of dysplastic cells in the remaining two tumor samples (D3 and D12) precluded mutation determination by sequencing, a technique which is less sensitive than the IVSP assay. Analysis of adjacent normal mucosa indicated that all of the identified mutations were somatic.

Alterations of the K-RAS gene in these polyps were next examined using a modified ligation assay which detects all possible activating mutations at codons 12 and 13b. This analysis detects 90% of the RAS mutations previously identified in colorectal tumors (19, 20). Mutations of K-RAS were equally abundant in dysplastic and nondysplastic polyps and were found in 5 of 22 (23%) hyperplastic polyps and 3 of 12 (25%) dysplastic polyps (Table 3). These results suggested that APC but not K-RAS mutations were closely associated with the advent of dysplasia.

To extend these observations to the smallest detectable lesions, 20 ACF were identified by methylene blue staining (10) and isolated by cryostat sectioning. Only 1 of the 20 ACF had histopathological changes consistent with dysplasia. Two separate APC gene mutations were identified in this dysplastic focus (Fig. 1B) but none were identified in the 19 nondysplastic lesions (Table 1; Fig. 1B). Strikingly, mutations of K-RAS were identified in all of the 19 (100%) nondysplastic ACF, while the single dysplastic ACF did not contain a K-RAS mutation (Table 3; Fig. 2).

Table 3 Mutations in colorectal lesions

Lesion type ^a	Dysplasia ^b	K-RAS mutations ^c (WT = 12Gly, 13Gly)	APC mutations ^d
Polyps			
H6	-	12Asp, 12Val	-
H13	-	12Asp	-
H14	-	12Asp	-
H16	-	12Asp	-
H20	-	12Asp	-
Adenomas			
D3	+	12Ala	+
D6	+	12Arg	+
D11	+	12Asp, 12Val	+
Aberrant crypt foci			
AC1	-	13Asp	-
AC2	-	12Val	-
AC3	-	12Asp	-
AC4	-	12Asp	-
AC5	-	12Asp	-
AC6	-	12Asp	-
AC7	-	13Asp	-
AC8	+	None	+
AC9	-	12Val	-
AC10	-	12Ala	-
AC11	-	12Asp	-
AC12	-	13Asp	-
AC13	-	12Cys	-
AC14	-	12Asp	-
AC15	-	12Val	-
AC16	-	12Val	-
AC17	-	12Val	-
AC18	-	13Asp	-
AC19	-	12Val	-
AC20	-	12Asp	-

^a Only polyps containing K-RAS mutations are listed, while all 20 ACF are listed.
^b The presence or absence of dysplasia is indicated by a + or -, respectively.
^c Activating K-RAS mutations were ascertained as described in "Materials and Methods" and indicated as the altered codon followed by the resulting amino acid.
^d APC mutations were ascertained as described in "Materials and Methods" and their presence or absence is indicated by a + or -, respectively.

Discussion

The findings reported here provide insights into the pathogenesis of the earliest stages of colorectal tumorigenesis. No APC mutations were identified in any of the 41 nondysplastic lesions examined, while such mutations were commonly observed (85%) in the 13 dysplastic lesions (12 polyps, 1 ACF). Because only a portion of the APC gene was evaluated, it is likely that virtually all of the dysplastic lesions had APC mutations. Furthermore, all of the dysplastic lesions examined were less than 6 mm in diameter and the smallest dysplastic lesion, an ACF, was composed of only a few dozen dysplastic crypts. The

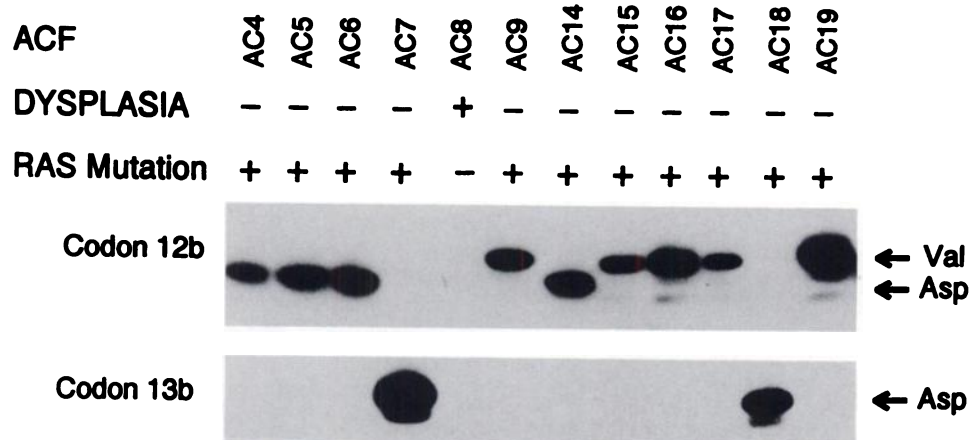


Fig. 2. K-RAS mutations in ACF. K-RAS mutations were detected using a mutation-specific ligation assay. Examples for 12 of the 20 aberrant crypt foci are shown. The presence of a K-RAS mutation is indicated by the presence of an appropriately sized ligation product. + or -, presence or absence, respectively, of dysplasia and K-RAS mutations.

results suggest that mutations of *APC* are closely associated with the advent of dysplasia, a hallmark of malignant potential. In contrast, mutations of *K-RAS* seemed to be independent of dysplasia. Moreover, the extremely high rate (19/19) of *K-RAS* mutations in nondysplastic ACF compared to either dysplastic or nondysplastic polyps suggests that such lesions are not the predominant precursor of either type of colorectal tumor. Recently, the discordance between the distribution of aberrant crypt foci and colonic tumors in dimethylhydrazine treated-mouse have led Carter *et al.* to suggest that ACF have little if any malignant potential in the mouse (21).

The above interpretation is consistent with previous data on *RAS* gene mutations. For example, *K-RAS* mutations were found in approximately 50% of dysplastic polyps larger than 1.0 cm but in only about 9% of smaller adenomas (9). Moreover, detailed analysis of *K-RAS* mutations in seven dysplastic polyps revealed that in four cases only a portion of the tumor cells contained a specific *K-RAS* mutation, indicating that *K-RAS* mutations often occur after tumor initiation (22). Likewise, two polyps were identified in the current study that contained more than one *K-RAS* mutation, suggesting that they arose independently, after polyp initiation. Finally, Pretlow *et al.* have found *K-RAS* codon 12 mutations in 73% of ACF but not in any of 27 morphologically normal crypt areas (23). Altogether, the data suggest a model where if a *RAS* gene mutation occurs as the first genetic event, a nondysplastic ACF will form which has little potential to progress. In contrast, if an *APC* mutation occurs first, a dysplastic ACF will result. Dysplastic ACF have the capacity to progress and this progression is driven by subsequent mutations in *RAS* and other genes.

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