Advances in Brief

Adenomatous Polyposis Coli Gene Mutations in Ulcerative Colitis-associated Dysplasias and Cancers *versus* Sporadic Colon Neoplasms[1]

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Abstract

Adenomatous polyposis coli (APC) gene mutations occur in most sporadic colonic adenomas and carcinomas. Precursor lesions of ulcerative colitis (UC)-associated colon carcinomas, although morphologically similar to sporadic adenomas, may be biologically distinct from them and are, in fact, managed differently. Since sporadic adenomas may also occur in UC, a method of discriminating between these forms of neoplasia could have clinical utility. We examined 33 patients with UC-associated dysplasias and cancers and 23 sporadic colon neoplasms in a side-by-side comparison for APC mutations. Codons 686–1693, containing 64% of all reported APC mutations (the mutation cluster region), were screened for truncating mutations using an *in vitro* synthesized protein assay. Two of thirty-three patients (6%) with UC-associated dysplasias and cancers had a total of three truncating APC mutations, all in frank carcinomas, while 17 of 23 (74%) with sporadic colonic neoplasms had mutations. DNA sequencing confirmed two mutations in codon 1460, replacing arginine with a stop codon, as well as one 2-base pair deletion, resulting in a frameshift and a stop at codon 1477. One specimen contained one each of these APC mutations. This apparent contrast in mutation rates at the mutation cluster region of APC is consistent with other biological characteristics separating sporadic colon neoplasms from UC-associated dysplasias and cancers. These data raise the possibility that nonadenomatous UC dysplasias may arise by a molecular pathway distinct from that prevailing in sporadic colon carcinogenesis, and they suggest a molecular assay to discriminate between sporadic adenomas and dysplasias occurring in UC.

Introduction

UC[3] is characterized by an increased propensity to the development of colonic carcinoma (1). Dysplasia occurring in UC constitutes a precursor of carcinoma and an indication for close surveillance or colectomy. However, proper management of dysplasia in UC patients is problematic because of sampling error, objectivity in diagnosis, and lack of consensus as to the timing of colectomy.

The molecular pathogenesis of UC-associated neoplasia has been studied for a number of years. Abnormalities reported in UCDCs include rare point mutations in *ras* family proto-oncogenes (2); LOH at the *p53, Rb,* and *MCC/APC* loci (3); and microsatellite instability (4). Recently, two APC protein truncations were reported among six patients with UC-associated dysplasia (5).

It is likely that an accumulation of multiple mutations leads to dysplasia and cancer in both UC and sporadic colonic malignancy (6). However, biological features differ between these two types of neo-

plasia. For example, mutations in *ras* proto-oncogenes are somewhat rarer in UC (2) than in sporadic colorectal cancers and adenomas (7). Moreover, the time interval between the diagnosis of sporadic adenoma and carcinoma usually exceeds the interval separating UC-associated dysplasia and cancer. Another distinction is the frequent occurrence of *p53* mutation in early (low-grade) dysplastic UC lesions (8), whereas *p53* mutation is rare in early dysplastic adenomas (9). Perhaps the most important contrast is the consistent ease with which sporadic colon polyps can be detected, whereas flat UC-dysplasias are often difficult to distinguish from their nondysplastic surroundings.

Germline mutations of APC cause familial adenomatous polyposis, and somatic APC mutations occur in most sporadic colorectal carcinomas (10–15) as well as in some cancers of the stomach (16, 17), pancreas (18), thyroid (19), ovary (20), and other primary sites. APC mutation is rare in esophageal cancers (21). The APC gene is located on chromosome 5q21 (7). This gene contains 8535 bp of coding sequence in 15 exons (11), and the protein product consists of 2844 amino acids with a molecular weight of 311,800 (12). More than 60% of all reported somatic mutations of APC are found in the upstream half of exon 15, referred to as the mutation cluster region (13). In addition, the majority of APC mutations described thus far result in truncation of its protein product (10–18). Reported mutations include either premature stop codons or frameshift mutations leading to premature stop codons downstream (12).

Previous studies have shown no significant differences in APC mutation rates between sporadic colon adenomas and carcinomas (6, 13). APC mutations have been detected in benign tumors smaller than 0.5 cm (22). There is also extensive similarity in the character of APC mutations between sporadic colorectal adenomas and cancers (7). For these and other reasons, it has been hypothesized that APC mutation constitutes an early event in colorectal carcinogenesis, perhaps contributing to adenoma formation.

Given the large size of the APC gene, direct sequencing is a laborious method of screening for mutations. Fortunately, Powell *et al.* (23) devised a method of screening for premature protein truncation mutations using *in vitro* transcription and translation of PCR products (23). Using this method, we examined 33 patients with a total of 51 UCDCs, along with 23 patients, each possessing one sporadic colonic neoplasm, for APC mutation in a side-by-side comparison. Results revealed a marked contrast between sporadic neoplasms and UCDCs regarding mutation rates within the mutation cluster region of APC.

Recent studies have demonstrated LOH on chromosome 9p in a variety of tumors, but it is relatively uncommon in sporadic colorectal carcinoma (24). In a separate arm of the current study, we analyzed patients with UCDCs to determine the prevalence of LOH on chromosome 9p.

Materials and Methods

Tissue Samples. Tissue samples were obtained at surgery from patients with known UC who developed colonic adenocarcinoma, flat dysplasia, and/or dysplasia-associated lesions or masses. Normal tissue was collected from normal lymph nodes or normal ileal mucosa in all patients. Microdissection was performed in 31 ethanol-fixed, paraffin-embedded specimens, resulting in a neoplastic epithelial cell percentage exceeding 70% (25). In the remaining 20 freshly frozen samples, gross cryostat dissection was limited to areas consisting mostly of neoplastic cells (25). The mutational screening method we used detects mutations present in less than 10% of cells analyzed (see below). Patients with sporadic colon cancer not associated with UC were studied in parallel as a positive control group; paired tumor and normal tissues were available from all members of this group.

DNA Extraction. DNA was extracted using a modification of the method of Goelz et al. (26) as described previously (27, 28).

In Vitro Synthesized Protein Assay. Codons 686–1693 from exon 15 of the APC gene were analyzed using an in vitro synthesized protein assay (21). These codons were chosen based on previous studies localizing approximately two-thirds of all APC mutations to this region. Either of two overlapping segments (designated segments 2 and 3) of APC, spanning codons 686–1217 or 1099–1693, was separately amplified using PCR as follows. Genomic DNA (100–200 ng) and 350 ng of each appropriate primer were added to a PCR reaction containing 0.25 unit of Taq polymerase (Promega, Madison, WI) in 10 mM Tris-HCl (pH 8.6) with 1.5 mM $MgCl_2$ and 0.2 mM concentrations of each deoxynucleotide triphosphate. Primers used for segment 2 (codons 686–1217) were 5'-[T7-trans]-ATGCATGTGGAACTTTGTGG-3' and 5'-GAGGATC-CATTAGATGAAGGTGTGGACG-3'; and for segment 3 (codons 1099–1963), they were 5'-[T7-trans]-TTTCTTCCATACAGGTCACGG-3' and 5'-GGAGGATCCTGTAGGAATGGTATCTCG-3'. The T7-trans motif, which contains the Kozak translation and T7 RNA polymerase consensus sequences, consisted of 5'-GGATCCTAATACGACTCACTATAGGGAGACCACCAT-GG-3'. Amplification conditions comprised 35 cycles of a 40-s denaturation (95°C), a 90-s annealing (59°C for both segments), and a 90-s extension (72°C). All PCR reactions included a 5-min extension period (72°C) after the 35th cycle. A 5-μl aliquot of each PCR reaction was used directly in a coupled transcription and translation reaction (TnT Kit; Promega) containing 5 μCi of [^{35}S]methionine translabel (Amersham/USB, Cleveland, OH) for 1.5 h at 30°C. Samples were then diluted with sample buffer and boiled for 5 min; 5 μl were analyzed on a 10% SDS-polyacrylamide gel. Proteins were visualized by autoradiography after impregnating the gel with Enhance (Amersham/USB) for 30 min and drying at 65°C for 2 h. In other experiments, this method detects mutant DNA species comprising less than 10% of the total DNA (21).

DNA Sequence Analysis. DNA sequencing was performed directly on PCR products yielding truncated proteins. PCR products were precipitated and used as sequencing templates. An internal primer was chosen based on the estimated size of the truncated protein product and added in a reaction using the Sequenase PCR Product Sequencing Kit (USB) following the manufacturer's protocol for internal labeling with [^{35}S]dATP. An aliquot of each sequencing reaction was analyzed by electrophoresis on denaturing polyacrylamide gels, followed by autoradiography.

LOH in UCDCs. PCR was used to amplify portions of the chromosomal region spanning 9p13–9p22. Oligonucleotide primer pairs were synthesized for the following six anonymous DNA markers, with chromosomal localizations given in parentheses: D9S162 (9p22–p23), D9S163 (9p21–q21), D9S126 (9p21), D9S104 (9p21), D9S171 (9p21), and D9S165 (9p21–q21); and the IFNA locus (9p22). Multiplex PCRs were performed (29). Between 40 and 50 ng of genomic DNA were amplified in a 10-μl reaction containing 0.25 unit of Taq polymerase in 10 mM Tris-HCl (pH 8.6) with 1.5 mM $MgCl_2$, 0.2 mM concentrations of each deoxynucleotide triphosphate, and 0.2 μCi of [^{32}P]dCTP. PCR products were denatured in 95% formamide, electrophoresed on 6% denaturing polyacrylamide gels, and visualized by autoradiography. LOH was defined as a visible absence or a greater than 50% reduction in the signal of one allele in the tumor compared to matching normal tissue. 9p-LOH studies were not performed on sporadic colorectal lesions, since these have been reported previously (24) and because this comparison was not the purpose of our study.

Results

Thirty-three patients with UCDCs and 23 patients with sporadic colorectal neoplasms were analyzed, containing a total of 51 lesions (18 dysplasias and 33 carcinomas). Genomic DNAs derived from surgical specimens were amplified from two portions of the APC gene, which were then used in an in vitro synthesized protein assay to detect truncating mutations (Fig. 1). Truncating mutations appeared as aberrantly migrating bands (Fig. 1).

Only 2 of 33 patients (6%) with UCDCs had truncating mutations. Three mutations were found; all were observed in frank carcinomas, one of which contained two different mutations (one on each allele). All were seen in APC segment 3. This low frequency contrasted markedly with the 17 of 23 (74%) sporadic colon cancers that exhibited truncating mutations of the APC gene. Among the sporadic colon tumors, 4 of 23 (17%) mutations were in APC segment 2 (17%), while 16 of 23 (70%) were in segment 3. Three sporadic tumors contained dual mutations, one in each segment (Fig. 1). In UCDCs, direct sequencing confirmed 2 mutations in codon 1460, replacing arginine with a stop codon, as well as one 2-base pair microdeletion leading to a frameshift and stop at codon 1477 (Fig. 2; Table 1). Tumor 113T contained one of each mutation.
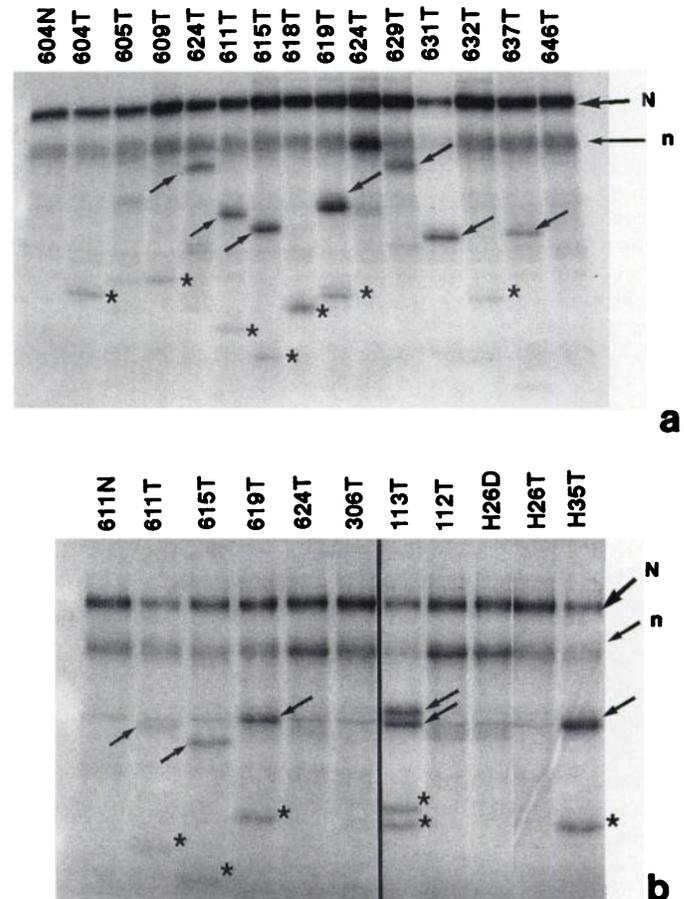

Fig. 1. a, truncating APC mutations in sporadic colorectal tumors. N (thick horizontal arrow), normal (full-length) protein band. n (thin horizontal arrow), nonspecific band. Angled arrows, truncated (mutant) proteins. *, consistent artifactual bands, possibly caused by internal ATG initiation sites. Note that most of these sporadic tumors contain mutations. b, Truncating APC mutations in sporadic tumors analyzed side-by-side with UCDCs. N, normal protein band. n, nonspecific band. Dark arrows, truncated proteins. *, bands possibly caused by internal ATG initiation codons. UCDC cases 113T and H35T exhibited truncated proteins, whereas the remainder of the UCDCs were negative for mutation. Note that 113T has two truncated bands.

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Nine of 31 informative patients (29%) with UCDCs exhibited LOH at one or more loci on chromosome 9p. LOH occurred in 16 of 54 lesions from these 31 patients (4 of 18 dysplasias and 12 of 36 carcinomas). However, DNAs from 16 of these lesions were not successfully PCR amplified at all 7 loci analyzed; therefore, these percentages could represent conservative estimates of the true prevalence of 9p-LOH in UCDCs (Fig. 3). Examples of 9p-LOH are shown in Fig. 3.

Discussion

APC is considered the "gatekeeper" gene in sporadic colon carcinogenesis as well as in some hereditary colorectal cancer syndromes (7). Sporadic colorectal cancers originate from polypoid adenomas. In contrast, colon cancers associated with UC arise from dysplasias that are often more difficult to distinguish from their nondysplastic surroundings. In addition, the natural history of sporadic adenomas suggests a prolonged progression time of 15 years before advancement to cancer occurs, whereas the typical dysplasias of UC progress more rapidly, usually within several years (29). Thus, the biological behavior of the two types of cancer differs. We studied APC mutation in 33 patients with UCDCs in parallel with a group of sporadic colon cancers. Our results revealed a marked contrast between the two types of cancer, with 6% of the UCDCs versus 74% of the sporadic tumors showing APC mutation. This latter percentage is consistent with previously published reports of sporadic colonic cancer (7).

This contrast in mutation rates at the mutation cluster region of APC is consistent with other biological differences between the two types of colon cancer. For example, numerous studies showed disparate ras mutation frequencies between sporadic adenomas and UCDCs (2, 5, 30, 31). However, not all molecular abnormalities differ between the two varieties of cancer. The prevalence of p53 tumor suppressor gene mutations is roughly equivalent, although the timing of these alterations may be earlier in UCDCs than in sporadic tumors (30, 31). In an analogous fashion, LOH has been shown to involve very similar loci in the two types of cancer (3, 5, 30, 31). Chromosomal regions frequently affected by LOH include 5q, 13q, 17p, and 18q. A 9p-LOH prevalence rate of 24% has been reported previously

Table 1 APC mutations in UC-associated neoplastic lesions

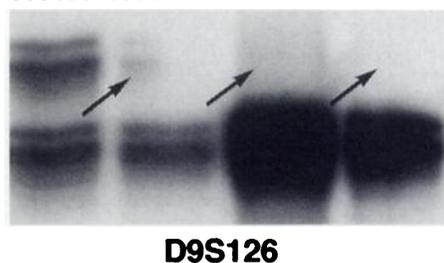| Sample	Codon	DNA mutation	Amino acid change
H35T	1460	CGA → TGA	Arg → stop
113T	1460	CGA → TGA	Arg → stop
	1477	2-bp deletion	Frameshift mutation → stop

Fig. 3. LOH on chromosome 9p occurring in ulcerative colitis. In patient H11, LOH was seen in three discrete tumors from different locations (angled arrows).

in sporadic colon cancer (24). This is in agreement with our results of 29% LOH in UCDCs. Thus, this locus is likely to be of limited importance in both types of cancer, despite its apparent involvement in a wide variety of other tumor types. A slight preponderance of LOH at several chromosomal loci has been reported in right colonic lesions for both UCDCs and sporadic colorectal carcinomas (3, 7, 30, 32). p53 mutation is also more common in proximal colonic lesions of both types. Similarly, microsatellite instability tends to be more common in proximal colon cancers in both UC and sporadic cases (4, 33–36).

We cannot exclude the possibility that mutations occurred in other unusual regions of APC in UCDCs, because we did not study these regions. Moreover, we cannot rule out the possibility that an unusually high prevalence of missense substitutions occurred in UCDCs, because this type of mutation would not have been detected by the in vitro protein synthesis screening technique. Nevertheless, we favor the conclusion that these data support a clear difference between the molecular pathogenesis of the two types of colonic cancer. It is possible that the relative rarity of APC mutation in UCDCs contributes to their unique biology and morphological characteristics. Finally, these data raise the possibility of using the IVS assay to discriminate UCDCs from sporadic adenomas, which may occur fortuitously in UC but be difficult to distinguish microscopically (37). Thus, the finding of a dysplastic lesion with an APC mutation might permit more conservative management than a lesion lacking mutation. These possibilities imply a need for future large prospective studies exploring the prevalence and natural history of APC mutation-positive versus mutation-negative dysplasias occurring in the setting of UC.

Acknowledgments

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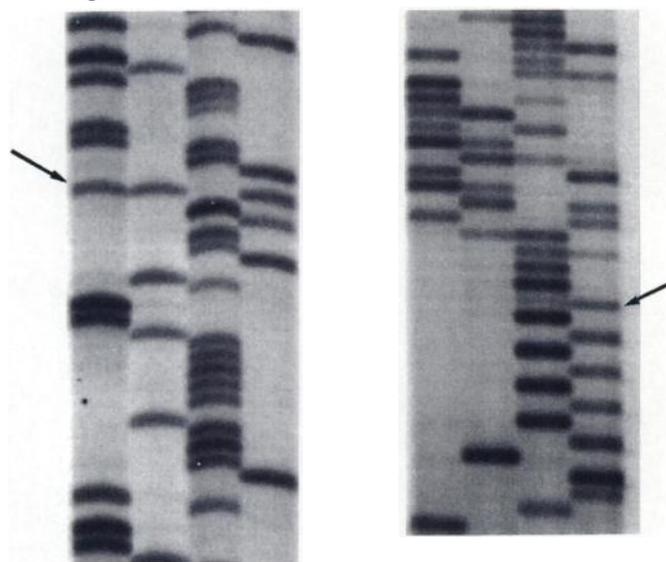

Fig. 2. Direct sequencing of PCR products showing truncated bands in Fig. 1. The noncoding strand is shown. Sample H35T contains a coding strand base substitution from C to T, while in 113T, there is a 2-bp deletion leading to a frameshift mutation (angled arrows).

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