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Identification of APC Gene Mutations in Italian Adenomatous Polyposis Coli Patients by PCR-SSCP Analysis

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Summary

The APC gene is a putative human tumor-suppressor gene responsible for adenomatous polyposis coli (APC), an inherited, autosomal dominant predisposition to colon cancer. It is also implicated in the development of sporadic colorectal tumors. The characterization of APC gene mutations in APC patients is clinically important because DNA-based tests can be applied for presymptomatic diagnosis once a specific mutation has been identified in a family. Moreover, the identification of the spectrum of APC gene mutations in patients is of great interest in the study of the biological properties of the APC gene product. We analyzed the entire coding region of the APC gene by the PCR–single-strand conformation polymorphism method in 42 unrelated Italian APC patients. Mutations were found in 12 cases. These consist of small (5–14 bp) base-pair deletions leading to frameshifts; all are localized within exon 15. Two of these deletions, a 5-bp deletion at position 3183–3187 and a 5-bp deletion at position 3926–3930, are present in 3/42 and 7/42 cases of our series, respectively, indicating the presence of mutational hot spots at these two sites.

Introduction

Adenomatous polyposis coli (APC) is a rare, dominantly inherited predisposition to colon cancer in which affected individuals develop hundreds to thousands of adenomatous polyps in the colon and rectum. Most APC patients, if left untreated, will develop colorectal cancer by the age of 40 years. It is important to diagnose the disease in the presymptomatic stage, as symptoms often indicate the development of cancer. After colorectal cancer, the major risks of death from this disease include upper-gastrointestinal cancer, desmoid tumor, and a number of other malignancies throughout the body. The exact magnitude of the risk of malignant degeneration for these extracolonic manifestations is as yet uncertain (Burt 1992).

Recently, the APC gene was isolated by positional cloning methods (Groden et al. 1991; Joslyn et al. 1991; Kinzler et al. 1991; Nishisho et al. 1991). This has enhanced the power of genetic analysis in counseling families, for it is now feasible to identify specific mutations in affected individuals and to follow these mutations in families.

Furthermore, the molecular characterization of the mutations is of great interest in the study of the biological properties of the APC gene product. Somatic mutations of the APC gene also have been detected in sporadic colorectal carcinomas (Nishisho et al. 1991; Powell et al. 1992). Loss of heterozygosity in the 5q21-22 region, where the APC gene is located, was found in 40%–60% of colorectal tumors (Solomon et al. 1987). Therefore, this gene is considered to be a tumor-suppressor gene that plays a significant and rate-limiting role in colorectal tumorigenesis.

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We have analyzed the APC gene by the PCR–single-strand conformation polymorphism (PCR-SSCP) analysis (Orita et al. 1989) in 42 Italian APC patients. The identification of mutations in 12 cases is reported here.

Patients, Material, and Methods

Identification and Clinical Characteristic of APC Patients

APC patients were identified through referrals associated with the diagnosis and treatment of APC, from hospital clinics. Medical records were obtained and reviewed to verify the diagnosis of APC in patients not directly followed by the authors. A total of 42 unrelated APC patients were selected on the basis of their availability and their willigness to donate blood samples. Age at diagnosis varied from 11 to 57 years (mean age at diagnosis was 33.7 years in symptomatic patients and 23.7 years in patients who underwent colonoscopy for familial screening).

Extracolonic manifestations were present in several patients and included the following: CHRPE (congenital hypertrophy of retinal pigmented epithelium) (81%), epidermoid cysts (31%), osteomas (38%), desmoids (13.7%), and upper-gastrointestinal polyps (30.5%). A set of 40 healthy individuals (blood donors) were used as a control.

Tissue Culture

Epstein-Barr virus–transformed lymphoblastoid lines were derived from patient and normal blood samples (Zeuthen 1983). These cell lines were cultured at 37°C in RPMI 1640 medium (Seromed) with the following supplements: 10% FCS, 2 mM L-glutamine, 1 mM sodium pyruvate, 100 μM nonessential amino acids.

DNA Extraction

DNA was obtained directly from peripheral blood or from lymphoblastoid cell lines by using standard methods (Sambrook et al. 1989).

PCR Amplification

Oligonucleotide primer pairs used for the PCR-SSCP analysis have been described by Groden et al. (1991, 1993). DNA samples were amplified using PCR (4 min at 94°C, once; 30 s at 94°C, 30 s at annealing temperature, and 30 s at 72°C, 30 times; and 7 min at 72°C, once).

Twenty-microliter PCR reactions (200 ng of DNA, 0.5 μM each primer, 70 μM each deoxyribonucleoside triphosphate, 1.5 mM $MgCl_2$, 0.25 U *Taq* polymerase [Biotech], 1 × reaction buffer [Biotech], 0.25 mM spermidine, 0.1 μl of 3,000 Ci [α-^{32}P]dCTP/mmol) with no mineral oil were run on a PCR 9600 Perkin Elmer Cetus machine.

SSCP Gel Analysis

PCR products were diluted in 50 μl of 0.1% SDS, 10 mM EDTA and 50 μl of 95% formamide, 20 mM EDTA, 0.05% bromophenol blue, 0.05% xylene cyanol. Products were denatured at 95°C for 4 min and were kept on ice until loaded onto polyacrylamide gels. Each set of reactions was analyzed using two gel conditions: (1) 5% acrylamide, 90 mM Tris-borate (pH 7.5), 2 mM EDTA at 4°C and (2) 5% acrylamide, 90 mM Tris-borate (pH 7.5), 2 mM EDTA, 10% glycerol at room temperature and with a fan blowing in front of the gel. Electrophoresis was carried out at 30 or 40 W, with constant power in both conditions (30 W was used when gels were run on an old, homemade apparatus). After electrophoresis, gels were dried on vacuum slab dryers and were exposed overnight, without intensifying screens, with Kodak X-Omat AR film.

Sequencing of SSCP Conformers

SSCP bands were cut from dried gels and were placed in 100 μl of distilled water. After shaking for 30 min and a brief centrifugation step, a 10-μl aliquot was used for PCR amplification with the same primer pair that was used in the original PCR reaction. Samples were purified by a Centricon 100 column (Amicon). Samples were sequenced with the dideoxy termination method with *Taq* polymerase and fluorescently tagged M13 universal or reverse sequencing primers on the Applied Biosystems model 373A DNA sequencer (Applied BioSystems, Foster City, CA).

Results

Detection and Characterization of SSCP Abnormal Conformers

The entire coding region (8,532 bp) of the APC gene was analyzed by the PCR-SSCP method in 42 unrelated APC patients. Primer pairs flanking each exon were used for exons 1–14, while exon 15 was examined with 23 overlapping primer sets (Groden et al. 1991, 1993). Forty normal individuals were screened each time bands of altered mobility were seen in the patient set. Abnormal SSCP conformers unique to patients were observed in 12 cases, with the following primer pairs: 15A (patient 1395), 15D (patient 1328), 15E (patients 1223, 1245, and 858), and 15G (patients 1382, 1346, 1332, 1221, 862, 1335, and 1387) (fig. 1).

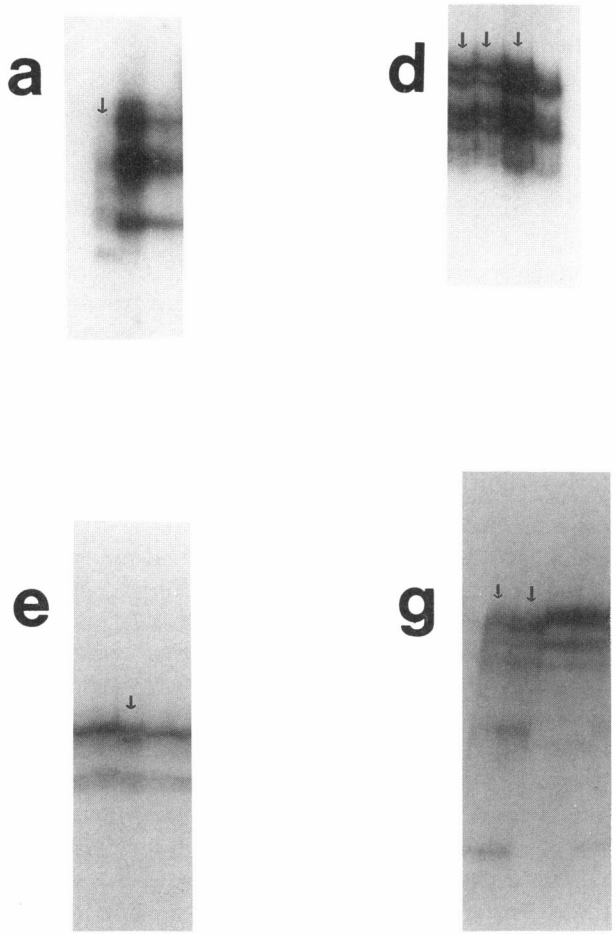

Figure 1 SSCP conformers specific to APC patients. Each panel shows one of the unique SSCP conformers found in 12 unrelated APC patients. Arrows mark the lanes containing the new conformers. The flanking lanes in each panel show the expected pattern of conformers in DNA from other affected individuals and from normal controls. The PCR products analyzed are from exon 15A (*a*), D (*d*), E (*e*), and G (*g*).

The sequence analysis of these novel bands revealed, in each case, the presence of small deletions disrupting the gene. A deletion of the first 14 bp of exon 15—AA ATC CTA AGA GAG (position 1961–1974)—was observed in patient 1395. This deletion caused a frameshift and a termination at position 2016–2018, because of the formation of a stop codon (TAG). Patient 1328 showed an 8-bp deletion—AT TTCACT (position 2807–2814)—that also introduced a stop codon (TAG) in the reading frame, at position 2832–2834. A 5-bp deletion—A CAA A (position 3183–3187)—was observed in patients 858, 1245, and 1223. This deletion created a stop codon (TGA) at position 3189–3191. A 5-bp deletion—AA AAG (position 3926–3930)—was detected in patients 1382, 1346, 1332, 1221, 862, 1335, and 1387. The deletion created a stop codon (TAG) at position 3939–3941. Each of these deletions could also be seen on an acrylamide-urea denaturing gel when PCR products from these deletion patients were electrophoresed along with PCR products from control individuals and with product from an M13 sequencing reaction (data not shown). Position 3926 (GAA→Glu) was found to be different from the published sequence (GGA→Gly) (Joslyn et al. 1991; Kinzler et al. 1991), in all the normal samples we analyzed. This seems to be a sequencing error, as North American APC patients have the same nucleotide substitution (Groden et al. 1993).

Confirmation of the Mutation in Affected Family Members

Material from other affected family members was available in some cases (858, 1221, 1332, 1328, 1387, and 1245). All affected members within these six families also showed the same abnormal SSCP pattern. Nine affected individuals from the kindred of patient 1346 were analyzed and found to carry the same deletion (data not shown).

Discussion

The APC gene recently has been isolated by positional cloning methods and has been identified as the gene responsible for APC, by the presence of germ-line mutations in some APC patients (Groden et al. 1991; Nishisho et al. 1991). Some of these mutations also were shown to segregate with the disease (Groden et al. 1991; Nishisho et al. 1991). The data presented here confirm this observation, as 12/42 (28.5%) unrelated Italian APC patients also carry germ-line mutation of APC. All the mutations reported here are small (5–14 bp) deletions located within exon 15, a 6.5-kb exon encoding 77% of the gene product . The deletion of A CAA A at position 3183–3187 was present in 3/42 cases of our patient set and has been observed also in other APC patients (4/79 in Miyoshi et al. 1992 and 3/60 in Groden et al. 1993). Similarly, the deletion of AA AAG at position 3926–3930 was detected in 7/42 of our patients and also has been observed in other mutational series (10/79 in Miyoshi et al. 1992 and 5/60 in Groden et al. 1993).

The presence of a common progenitor in our cases seems unlikely, as these APC chromosomes carry different alleles at the highly polymorphic locus D5S346, tightly linked to the APC gene (Spirio et al. 1991). In

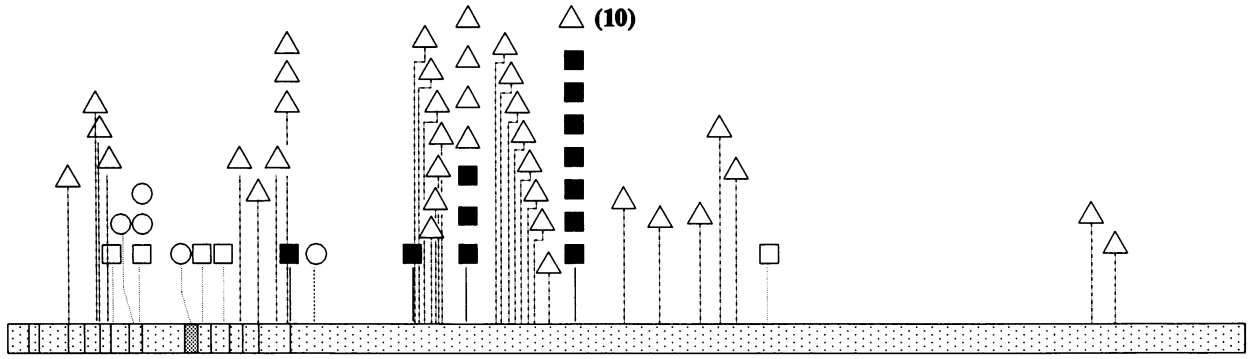

Figure 2 Coding region of the APC gene, shown accurately reflecting the proportion of each exon. Different symbols indicate APC mutations described in patients, by different studies: Groden et al. (1991) (□), Kinzler et al. (1991) (○), present study (■), and Miyoshi et al. (1992) (△).

addition, the ACAAA deletion of patient 1223 is a new mutational event, as neither parent carried this mutation. These two mutations account for 20% of the APC gene mutations now described in APC patients (fig. 2). Therefore we think these sites should be considered mutational hot spots. The presence of homopolymeric runs of bases at both sites (AAATAAA[ACAAA] and AAATAAAAG[AAAAG], respectively) could favor DNA polymerase errors.

The PCR-SSCP method of mutation detection (Orita et al. 1989) has been a fast and simple technique for the initial screening of the long coding sequence (8,532 bp) of the APC gene. According to other authors, two factors determine the sensitivity of this method: (1) the running conditions of the polyacrylamide gels and (2) the length of the analyzed PCR products. Hayashi (1991) estimates that the sensitivity of this method varies from 92% (100–300-bp fragments) to 75% (300–450-bp fragments). Sarkar et al. (1992) tested the sensitivity of SSCP for small (183-bp) and large (307-bp) segments at different running conditions. They found that the length of the PCR product greatly affected the efficiency of mutations detection (75%–92% for the 183-bp product and 50%–64% for the 307-bp product).

The results presented here (28.5% of mutations identified) seem to indicate a lower sensitivity of SSCP analysis, compared with either denaturing gradient gel electrophoresis (Fodde et al. 1992; Olschwang et al. 1993) or RNAse protection analysis (Miyoshi et al. 1992). However, the percentage of APC gene mutations found in sporadic colorectal adenomas (18/38 cases; L. Varesco, unpublished data) by using SSCP analysis is close to the results (10/16 cases) reported by Powell et al. (1992), using sequence analysis. Therefore, we suggest that (1) some mutations may not be seen by using these SSCP running conditions, (2) some mutations (e.g., deletion of entire exons) cannot be seen by the SSCP method, (3) some mutations could lie outside the examined coding region (i.e., mutations at the 5′ promoter region, at the 3′ untranslated region, and in the intronic regions), and (4) genetic heterogeneity may be present. Up to now, linkage studies have not demonstrated the existence of polyposis families unlinked to 5q markers, but this possibility cannot be excluded. Large deletions at the APC locus also have been described (Joslyn et al. 1991): pulsed-field gel electrophoresis analysis may be necessary to detect this kind of mutation.

The clinical characteristics of patients for whom a mutation has been identified are listed in table 1. Patients with the identical molecular alteration of the APC gene differ in the severity of clinically evident extracolonic disease manifestations (CHRPE, epidermoid cysts, desmoids, and upper-gastrointestinal polyps). The appearance of these features may be stochastic or related to other genetic or environmental factors. Presently, we are trying to obtain clinical information and molecular data in more individuals of our families, to test this hypothesis.

The APC mutations described so far in patients are all inactivating mutations expected to eliminate or to affect severely the function of the APC gene product. As information about the functional domains of the APC gene product is still lacking, the clinical significance of other types of variants, such as missense mutations, could be more questionable. Similarly, the role that milder APC mutations play in predisposition to "sporadic" colorectal neoplasia needs to be evaluated.

Table 1

Clinical Characteristics of Patients in Whom a Mutation Has Been Found

Patient	Mutation		Age (years)	Clinical Characteristic[a]					
	Type	Position		CHRPE	Epidermoid Cyst	Osteoma	Upper-Gastrointestinal Polyp	Desmoid	Other
1382	−AAAAG	3926	11[b]	NA	0	0	+	0	0
1346	−AAAAG	3926	11[c]	+	+	+	+	0	0
1332	−AAAAG	3926	16[b]	+	0	NA	0	0	0
1221	−AAAAG	3926	17[c]	+	0	+	0	0	0
862	−AAAAG	3926	23[b]	0	0	0	0	0	0
1335	−AAAAG	3926	19[b]	NA	+	NA	+	NA	Carcinoma thyroid
1387	−AAAAG	3926	NA	NA	NA	NA	NA	NA	NA
858	−ACAAA	3183	28[c]	NA	NA	+	0	NA	Lipoma
1245	−ACAAA	3183	26[b]	NA	+	+	+	0	0
1223	−ACAAA	3183	24[b]	+	+	+	0	0	0
1328	−ATTTCACT	2807	34[b]	+	0	0	NA	0	0
1395	−AAATCCTAAGAGAC	1961	NA	NA	NA	NA	NA	NA	NA

[a] The presence of extracolonic manifestation is scored as positive (+), negative (0), or NA (information not available).
[b] Diagnosis for symptoms.
[c] Diagnosis for familial risk screening.

In conclusion, we report the identification of specific mutations (small-base-pair deletions) of the APC gene in Italian APC patients. The finding of specific APC mutations in patients enables us to offer DNA-based tests for presymptomatic diagnosis to at-risk members of these families.

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