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Mutational Analysis of the First 14 Exons of the Adenomatous Polyposis Coli (APC) Gene

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In the present study, the polymerase chain reaction single strand conformation polymorphism (PCR-SSCP) technique has been applied to the mutation analysis of the adenomatous polyposis coli (APC) gene. We examined the first 14 exons of the APC gene in 46 polyposis coli patients. Five germline mutations were observed, including a single-nucleotide substitution and small (1–4 bp) deletions leading, in 4 cases, to a stop codon. A missense mutation in exon 3 and a 1 bp deletion in exon 4 of the APC gene were observed in patients presenting with the attenuated form of FAP.

Key words: APC gene, familial polyposis coli, genetics, mutation
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INTRODUCTION

FAMILIAL ADENOMATOUS polyposis (FAP) is a rare autosomal dominant inherited disorder predisposing to colorectal cancer. Patients with FAP develop hundreds to thousands of adenomatous polyps in the colon and rectum during their second or third decades of life which, if left untreated, will almost certainly develop into colorectal cancer. Recently, several reports have indicated the evidence of a phenotypically different form of polyposis coli [1–4] known as the attenuated or variant form of APC (AAPC). It is characterised by a low and variable number of colonic polyps (from only a few to over a hundred), a more advanced age of onset and/or slower evolution of the disease.

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Germline mutations in the APC gene are believed to be responsible for FAP. This gene was first localised to 5q21-22 and subsequently cloned [5–9]. The full length APC transcript consists of 15 exons, and encodes a protein of 2843 amino acids [9]. The coding region of the APC gene is preceded by several alternatively spliced 5′ non-coding exons [10].

Mutations in the APC gene have also been detected in sporadic carcinomas and adenomas [11–13]. The simultaneous existence of somatic and germline alterations of the APC gene have been observed in a high portion of desmoid tumours [14]. These results strongly suggest an important role for the APC gene product in epithelial proliferation and/or differentiation. Somatic mutations in APC have been suggested to initiate colorectal tumour development in the general population, whereas germline mutations result in FAP.

We have used the technique of single strand conformation polymorphism (SSCP) to determine the germline mutations in the first 14 exons of the APC gene in 46 unrelated Swiss polyposis coli patients. Identification of five mutations occurring in this region is reported here and discussed in the light of the patients' phenotypes.

MATERIALS AND METHODS

APC patients

Among 46 unrelated polyposis coli patients, there were 42 cases with the classical APC phenotype. 6 of these presented with extracolonic symptoms including osteomas (3 patients), desmoids (2 patients), lipomas (2 patients) and upper gastrointestinal polyps (3 patients). There were 4 individuals who presented with less than 100 colonic polyps at older ages (range 44–77 years) from which 2 had developed colon cancer. All 4 patients, however, have a family history of FAP or colorectal cancer. There were 8 polyposis cases with no previous family history of colorectal or upper gastrointestinal tract disorders. The selection of these cases was based on an early age of onset and the presence of an elevated number of polyps within the colon. All patients without a family history of FAP, except one, presented with symptoms typical of polyposis coli. The one exception presented with colonic polyposis and an astrocytoma indicative of Turcot's syndrome.

The age at which patients with a family history of FAP were diagnosed varied from 13 to 77 years with a mean 40.7 years. The mean age of diagnosis of patients without a family history of FAP was 34.5 years with a range 25–50 years.

DNA isolation

DNA was obtained from peripheral blood lymphocytes as previously described [15]. Briefly, whole blood was mixed with a three-fold volume of EC buffer (155 mM NH_4Cl, 10 mM $KHCO_3$, 0.1 mM EDTA) and incubated on ice for 15 min. The lysate was centrifuged and the pellet was resuspended in 3 ml SE buffer (75 mM NaCl, 25 mM EDTA, pH 8.0), 1% SDS, 20 μg/ml proteinase K and 10 μg/ml RNase and incubated overnight at 37°C. The next day the reaction mixture was diluted by an additional 3 ml of SE buffer and, to the resulting solution, 3 ml of 6 M NaCl was added and mixed to precipitate proteins. The total reaction mixture was then centrifuged and the DNA precipitated from the supernatant in a fresh tube, washed with 70% ethanol twice and once with 95% ethanol, dried and finally resuspended in 1 ml of TE buffer (10 mM Tris-Cl, pH 7.5, 0.1 mM EDTA).

PCR amplification

The polymerase chain reaction (PCR) was used to amplify genomic DNA. Primer sets used to amplify exons 1–14 of the APC gene were as described previously [9]. Ten microlitres of PCR reaction mixture contained 200 ng genomic DNA, 0.5 μM each primer, 0.25 μM each dNTP, 5 mM $MgCl_2$, 0.2 U Taq polymerase, 1x reaction buffer (10 mM Tris, 50 mM KCl, 0.2 mg/ml BSA, pH 8.5) and 0.1 μl of 3000 Ci (α-^{32}P) dCTP/mmol (Dupont, Boston, Massachusetts, U.S.A.). The reaction parameters were 1 min at 93°C, 30 s at 50°C and 1 min at 72°C for 19 cycles.

SSCP gel analysis

PCR products were diluted 1:10 in denaturing buffer (95% formamide, 20 mM EDTA, 0.05% bromphenol blue and 0.05% xylene cyanol), heated at 80°C for 2 min and applied to a 5% non-denaturing polyacrylamide gels. Electrophoresis was performed at 6 W constant power at 4°C for 8–9 h. Thereafter, the gels were dried and autoradiographed.

DNA sequencing

New SSCP conformers were further investigated by sequencing. DNA was amplified using the same primers as those for PCR-SSCP, purified using a QIAGEN Gel Extraction Kit (Qiagen Inc., Chatsworth, California, U.S.A.) and sequenced with the dideoxy chain-termination reaction using Sequenase Version 2.0 DNA Sequencing Kit (United States Biochemical Co., Cleveland, Ohio, U.S.A.) as per the manufacturer's instructions.

RESULTS

The coding region of exons 1–14 of the APC gene was analysed by the PCR-SSCP method in 46 unrelated FAP patients. Abnormal SSCP conformers unique to the patients were observed, and the sequencing analysis of these novel bands revealed, in each case, the precise site and character of the mutational change. A mutation in one of the first 14 exons of the APC gene was observed in 10.8% of cases (5/46). Only 5% of the total number of FAP cases with a previous family history had mutations in this region of the APC gene (Figure 1) whereas mutations of the same region were responsible for 3/8 of the cases without a family history of FAP (Figure 2).

All except one mutation reported here are small deletions (1–4 bp) or base-pair substitutions that result in a stop codon. One base-pair substitution, however, lead to an amino acid change. A summary of all mutations are listed in Table 1.

Patient A showed a base-pair substitution in exon 3 (codon 99) resulting in an arginine to tryptophan amino acid change. The patient is 60 years old and presents with symptoms indicative of FAP. However, there are estimated to be less than 100 polyps in her colon and she has not developed colorectal cancer. The patient comes from a family with a history of colorectal cancer, as her father and brother died of metastatic colon cancer, her grandmother died of stomach cancer and her sister has been operated on due to cancer of the rectum. The same APC mutation has also been found in patient's 38-year-old daughter. Colonoscopic examination, however, revealed no adenomatous polyps in her colon or rectum. No additional information concerning other affected family members is available. To provide evidence that this was not a common polymorphism, over 50 persons were investigated, all of whom were negative with respect to this polymorphism.

The 36-year-old patient B has a 1 bp deletion in codon 151 (exon 4) of the APC gene. This DNA mutation leads to the formation of a stop codon 6 bp later. The patient has not as yet developed colorectal cancer and, similarly to the previous patient, colonoscopic examination has revealed less than 100 polyps in his colon or rectum. Interestingly, the patient's father and aunt, who had the same mutation in the APC gene and presented with only a few colonic polyps (<100), died of colon carcinoma at the age of 45. In addition, 2 of 3 of the patient's offspring have inherited the mutation, but they are too young to have developed symptoms at present.

A deletion of 4 bp was detected in patient C at position 505–508 (codons 169–170) in exon 4 that leads, 12 bp downstream, to a stop codon. This 28 year old patient appears to harbour a new mutation, as there is no family history of disease.

The nucleotide change C-T at position 694 (codon 232, exon 6) was observed in patient D. This 25-year-old patient underwent colectomy. There is no family history of polyposis coli, therefore, we believe it to be a new case.

The final case of polyposis coli without a family history of disease, patient E, harbours a 1 bp deletion in codon 498 (exon 11), which results in a stop codon 21 nucleotides later. A total colectomy was performed on this 24-year-old patient as he

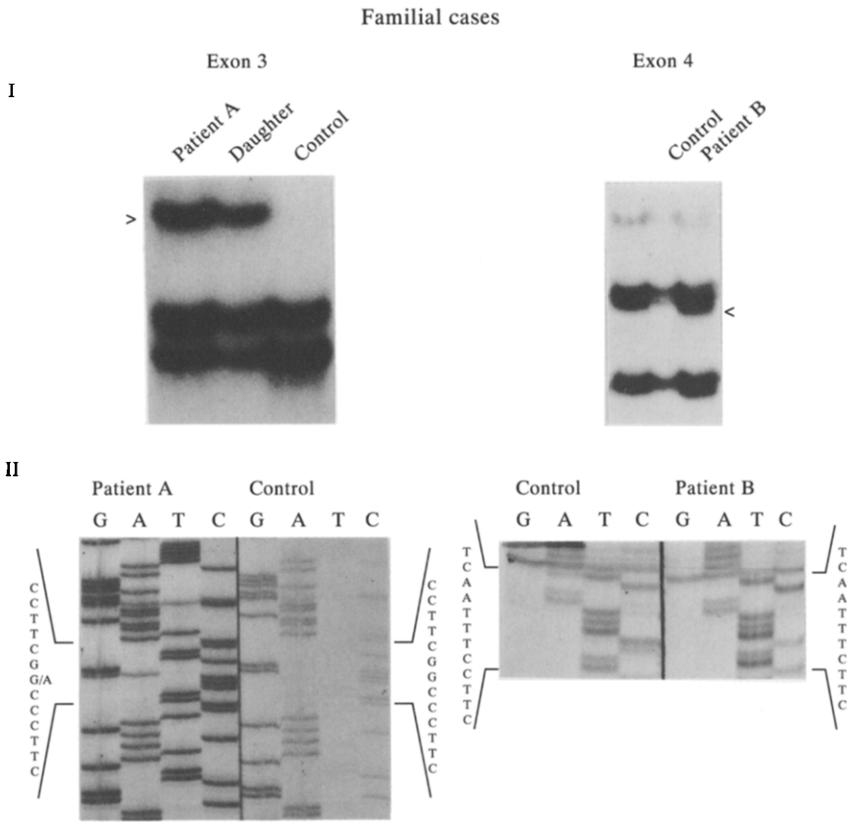

Figure 1. I. SSCP analysis of exons 3 and 4 from unrelated polyposis coli patients with a family history of FAP (A, B) and controls. The arrows mark the new SSCP conformers unique to these patients. II. Sequence analysis of SSCP conformers from the same patients and controls revealing the precise mutation. For patient A genomic DNA was used for sequencing whereas for patient B's sequence the corresponding SSCP conformer was eluted from the gel and sequenced directly.

had already developed metastatic disease from a colorectal carcinoma.

It is impossible to say if the mutations described for patients C, D and E represent new germline mutations as currently none of them have had children.

Finally, we have observed the polymorphism in exon 11, nucleotide 1458, described elsewhere [9]. The base-pair substitution T-C does not result in an amino acid change. In our set of 46 patients we identified 24 heterozygotes (52%) and 10 homozygotes (22%) of this polymorphism.

DISCUSSION

PCR-SSCP analysis of genomic DNA identifies most single- or multiple-base changes in DNA segments up to 400 bp long [16]. These changes are identified as shifts in single-stranded DNA conformers on non-denaturing polyacrylamide gels. It has been estimated that the sensitivity of this method reaches 96% for fragments of 100–200 bp, and decreases slightly for fragments of 200–300 bp [17].

Fifteen pairs of PCR primers were used to amplify exons 1–14 of the APC gene from DNA of 46 unrelated polyposis coli patients. Five different germline mutations were found, indicating that 10.8% of the patients carry the mutation in this region of the APC gene. This percentage is comparable with that observed by Miyoshi and colleagues [13], who screened 79 patients and found that 17.7% had mutations in this region using a RNAse protection assay, and Olschwang and associates [18], who observed 16% of 160 patients using the method of denaturing gradient gel electrophoresis (DGGE). Interestingly, Varesco and colleagues [19] did not find any mutation within this region among 42 unrelated FAP patients using the method of SSCP. From our results and those of others, we conclude that the mutation frequency in this section of the APC gene is not significantly higher than 20%.

Among the five different mutations presented here, there is one (exon 6, codon 232) which has already been published by several authors [13, 18, 20, 21]. This may represent a site that is more commonly mutated in the APC gene, however, further data accumulation is required for confirmation.

The only missense mutation found in this study causing an Arg-Trp amino acid change cannot be definitely assigned as a disease-causing mutation (rather than a rare polymorphism). It remains probable, however, since the mutation is of a non-conservative nature, and is located at position 295 in a region reportedly necessary for APC oligomerisation [22]. It has been proposed that the small number of APC missence mutations found is due to the lack of sufficient influence of these mutations on the protein to give rise to the FAP phenotype. However, as the physiological role of the APC protein is poorly understood, missense mutations, which segregate with the genetic disorder may be of value for assessing protein function, and therefore provide clues for localising the critical APC effector domains [13].

In the present study, no mutations were found in exons 1 and 2, in agreement with other studies describing a similar lack of mutations 5′ of exon 3 [13, 18, 19, 23, 24]. To date, most 5′

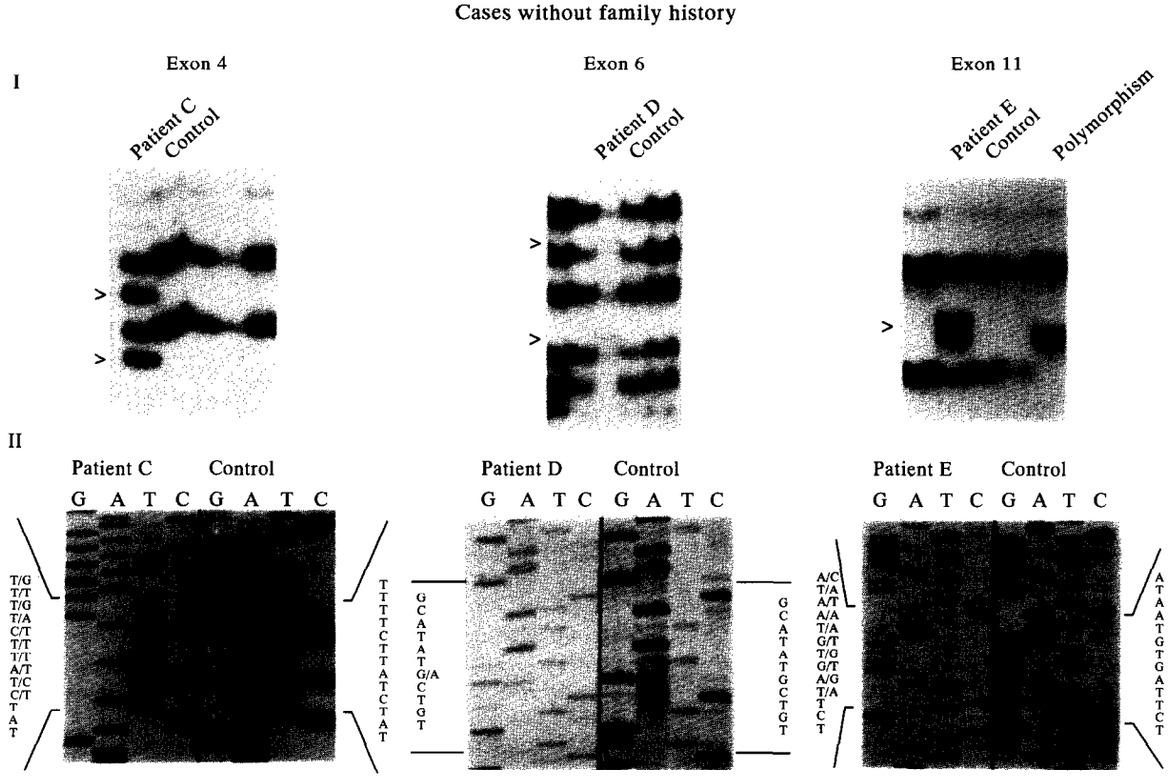

Figure 2. I. SSCP analysis of the exons 4, 6 and 11 from unrelated polyposis coli patients without a family history of disease (C, D, E) and controls. The arrows mark the new SSCP conformers unique for these patients. II. Sequence analysis of the conformers from the same patients and controls revealing the precise mutations.

Table 1. *Germline mutations in the first* 14 *exons of the APC gene*

Patient	Exon	Codon	NT change	Mutation	Stop codon
A	3	99	cgg-tgg	Arg-Trp	–
B	4	151	ttcctt-ttctt	Frameshift	6NT downstream
C	4	169–170	del (atag)	Frameshift	12NT downstream
D	6	232	cga-tga	Arg-Stop	Immediate
E	11	498	ctaag-ctag	Frameshift	21NT downstream

mutations were observed in kindreds where the FAP phenotype is attenuated [3], and they are clustered in exons 3 and 4 causing either a splice-donor defect or frameshifts. With respect to these observations, it has been proposed that a functional boundary within the APC gene exists between codons 157–168 that leads to either APC or AAPC depending on which side of this site the mutation lies.

Recently, Su and associates [22], using an *in vitro* expression system, confirmed APC dimerisation and showed that the first 171 residues of the APC protein are sufficient and the first 45 amino acids necessary for this oligomerisation. These results indicate that most mutant APC proteins should be able to bind to wild-type APC protein and inactivate it in a dominant negative manner. Only very small truncated proteins, resulting from the 5′ region mutations, are unable to associate with wild-type APC and, as such, cannot form a non-functional protein-dimer. The phenotype of patients with such mutations may, therefore, be milder than that of typical FAP patients, perhaps identical to the attenuated form of FAP.

A recent study excludes, however, the involvement of the APC gene in two kindreds affected by the attenuated form of FAP, and suggests the existence of another gene elsewhere in the genome, mutations of which give rise to a phenotype similar to FAP [4].

Patient A, who harbours an exon 3 mutation, displays a phenotype similar to FAP, although she presented with a lower number of colonic polyps and has not developed colorectal carcinoma, even though she is over 60 years of age. As her phenotype appears to be very mild, the attenuated form of FAP can be considered. The mutation found represents one of the closest to the 5′ end of the APC gene thus far described. It is located in the region assumed to be responsible for AAPC phenotype and supports the observations made by Spirio and colleagues [3]. The daughter of this patient was identified as carrying the same APC mutation. At the age of 38 years, however, she remains unaffected. In comparison, Spirio and associates [3] also observed heterogeneity in the number of adenomatous polyps, ranging from 0 to 100, found among

carriers of AAPC alleles. They identified 14 obligate carriers who have not developed any polyps in the colon or rectum, even though some have reached an advanced age. A possible explanation is another gene, perhaps at an unlinked modifier locus, which influences the heterogeneity, as has been suggested by studies in the multiple intestinal neoplasia (min) mouse [25, 26].

The possible correlation between the phenotype and the site of mutation in patient A and her daughter, however, can be associated with at least two hypotheses. Either the mutation located in this region interferes with APC homodimerisation, and therefore does not act in a dominant negative manner, or the missence mutation does not affect the APC protein absolutely with respect to its function. Both situations could result in a milder phenotype as the function of the APC protein may not be significantly altered.

Interestingly, patient B presents with the phenotype also considered as AAPC. The mutation at codon 151 lies within the region proposed by Spirio and colleagues [3], which gives rise to the AAPC phenotype (codons up to 157).

A mutation at codon 169–170 was found in patient C who presents with typical polyposis coli. Patients D and E, who have the mutations in exons 6 and 11, present also with expected symptoms.

In summary, the genotype/phenotype correlation between patients A and B provide further evidence that a functional boundary between codons 157–168 within the APC gene exists [3]. This is indirectly supported by the observation that the other diagnosed cases presented with features typical of FAP.

In conclusion, we report here the identification of specific germline mutations in the first 14 exons of the APC gene in a set of 46 polyposis coli patients. Taken together, the results tend to support the notion that FAP severity may be associated with the site of mutation within the APC gene. Furthermore, we observed that milder phenotypes tended to segregate with mutations at the 5′ end of the gene. Completion of the screening on the same series of patients should further identify mutational changes towards the 3′ end of the APC gene.

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