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Germline mutations of the *APC* gene in Korean familial adenomatous polyposis patients

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Abstract We extensively analyzed genomic DNA and messenger RNA (mRNA) from 62 unrelated Korean patients with familial adenomatous polyposis (FAP) for identification of germline *adenomatous polyposis coli* (*APC*) gene mutations. We adopted both single-strand conformation polymorphism (SSCP) analysis and a method of analysis involving the reverse transcription-polymerase chain reaction (RT-PCR) followed by a protein truncation test (PTT). DNA sequencing confirmed all alterations represented by aberrant bands. Germline mutations were identified in 38 patients (61%). Nineteen of the detected mutations were presumed to be novel, thus emphasizing the heterogeneity of the mutational spectrum in Korean FAP patients. In the initial 48 patients, SSCP analysis was followed by PTT for those patients for whom no detectable mutations were found by SSCP. Using this combined approach, we identified germline *APC* gene mutations in 29 of the 48 FAP patients (60%), including 6 patients in whom SSCP analysis failed to distinguish the mutant allele. In the 14 later patients, we identified truncating mutations in 9 patients (64%) using PTT only. Our results confirm that the mutation detection rate with PTT was superior to that with SSCP, and suggest that PTT would be a more practical screening method to detect germline mutations of the *APC* gene in FAP patients.

Key words Familial adenomatous polyposis (FAP) · *APC* gene · germline mutation · protein truncation test (PTT) · genetic testing

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Introduction

Familial adenomatous polyposis (FAP) is an autosomal dominantly inherited disease with almost complete penetrance. It is characterized by the development of hundreds to thousands of adenomatous polyps throughout the colon. Some of these polyps inevitably progress to colorectal carcinoma if prophylactic colectomy is not performed. In addition to colonic polyposis, FAP patients have a variable prevalence of extracolonic manifestations of both a benign and a malignant nature. Thus, FAP is considered a generalized growth disorder.

Traditional surveillance strategies for at-risk members of FAP families have depended on frequent endoscopic examinations, beginning at puberty. However, identification of the adenomatous polyposis coli (*APC*) gene responsible for FAP allowed the carrier status of family members to be determined (Groden et al. 1991; Kinzler et al. 1991; Nishisho et al. 1991). Thus, genetic testing for *APC* gene mutations in FAP patients and their family members is now regarded as an important part of management in FAP.

Since the identification of the *APC* gene, many different germline mutations have been reported. To date, germline mutations of the *APC* gene have been documented in about 500 FAP patients (Beroud and Soussi 1996; Gayther et al. 1994; Wallis et al. 1994; Dobbie et al. 1996; van der Luijt et al. 1997). The majority of germline mutations (over 95%) identified cause truncation of the *APC* protein.

With growing data on *APC* mutations and findings of biological significance in *APC* protein domains, the variations in FAP manifestations have been partly explained. Although many studies have shown both inter- and intrafamilial phenotypic variations among patients carrying the identical mutation (Groden et al. 1993; Paul et al. 1993; Varesco et al. 1993; Giardiello et al. 1994; van der Luijt et al. 1995; Scott et al. 1995), some studies have indicated clear genotype-phenotype correlations between the location of the *APC* gene mutation and the characteristic manifestations of FAP. These include the number of polyps (Nagase et al. 1992; Spirio et al. 1993; Gayther et al. 1994; Scott et al.

1995; Friedl et al. 1996), the age of onset and death (Nagase et al. 1992; Caspari et al. 1994; Gayther et al. 1994; Scott et al. 1995; Friedl et al. 1996), the presence of congenital hypertrophy of the retinal pigment epithelium (CHRPE) (Olschwang et al. 1993; Caspari et al. 1995), and the occurrence of desmoid tumors (Caspari et al. 1995; Davies et al. 1995; Scott et al. 1995; Dobbie et al. 1996; Eccles et al. 1996). Thus, the evidence strongly suggests that there are different functional mutations within the *APC* gene with respect to the position of the mutation.

Among the many techniques developed for the identification of diverse mutations in genetic disease, single-strand conformation polymorphism (SSCP) analysis has been widely employed for its simplicity (Orita et al. 1989). However, the large size of the *APC* coding region makes the detection of mutations labor-intensive and costly. The observation that the majority of mutations in the *APC* gene resulted in a truncated protein led to the development of a test based on the in-vitro translation, coupled with the transcription of polymerase chain reaction (PCR) products for selectively detecting translation-terminating mutations (Powell et al. 1993; van der Luijt et al. 1994). The protein truncation test (PTT) has a great advantage in that after only five separate PCR amplifications, the entire coding region of the *APC* gene is sufficiently covered.

To characterize germline mutations of the *APC* gene in 62 unrelated Korean FAP patients, we screened the entire coding region of the *APC* gene by SSCP analysis and PTT. In this study, we demonstrated the superiority of PTT in the detection of germline mutations in FAP patients. We also present the genotype-phenotype correlations detected in our patients.

Patients and methods

Patients and extraction of DNA and RNA

Blood samples and clinical information about 62 unrelated Korean FAP patients were obtained via the Korean Polyposis Registry, established in 1990. All 62 patients gave their informed consent prior to blood collection. Genomic DNA was extracted from the leukocytes of these patients by a salting-out procedure. Total cellular RNA isolation was performed in 20-ml aliquots of fresh peripheral blood samples, using a commercially available RNA/DNA/protein isolation reagent (Tri Reagent, Molecular Research Center, Cincinnati, OH, USA).

PCR and single-strand conformation polymorphism (SSCP)

Oligonucleotide primers were designed to amplify the entire coding region of the *APC* gene. Primer sets used to amplify *APC* exons 1–14 and the 17 overlapping segments of exon 15 have been described (Miyoshi et al. 1992). DNA samples were amplified for SSCP analysis using PCR under the following conditions: 5 min at 95°C; 1 min at 95°C, 1 min at 54°C, and 1 min at 72°C for 35 cycles; and 5 min at 72°C. Reaction mixtures, in a total volume of 25 μl, contained the following: 50 ng of DNA template, 0.4 μM each primer, 120 μM each dNTP, PCR buffer (10 mM Tris, 50 mM KCl, 1.5 mM MgCl2, 0.001% gelatin; Perkin Elmer Cetus, Foster City, CA, USA) 0.1 μCi/μl 32P dCTP (3000 Ci/mmol) (Amersham, Flylesbury, U.K.), and Taq DNA polymerase 0.01 U/μl (Perkin Elmer Cetus). After amplification, 15 μl of reaction mixture was diluted with of 15 μl of formamide dye (deionized formamide 95%, 10 mM ethylenediaminetetraacetate [EDTA], 0.05% xylene cyanol, and 0.05% bromphenolblue). Samples were denatured at 95°C for 4 min and placed on ice prior to being loaded into SSCP gel. For the optimum resolution in SSCP gel, large PCR products (more than 350 bp) were digested with appropriate restriction enzymes. Four μl of each reaction mixture was loaded into a 6% (48:1 acrylamide:bis) non-denaturing polyacrylamide gel. Electrophoresis was performed at 400 V constant voltage at 4°C for 13–16 h, or at 800 V constant voltage at room temperature for 2.5–3 h. Gels were blotted onto Whatman 3 MM paper (Maidstone, U.K.), covered with vinyl wrap, and then dried. Dried gels were exposed to Fuji RX film (Fuji Photo Film, Tokyo, Japan) or Kodak X-OMAT AR film (Eastman Kodak, Rochester, NY, USA) for 12–24 h.

Protein truncation test (PTT) and reverse transcription PCR (RT-PCR)

The full length of the *APC* gene was analyzed for mutations, using PTT as described previously (Powell et al. 1993; Roest et al. 1993; van der Luijt et al. 1994). The entire coding region of the *APC* gene was amplified in five overlapping segments by PCR, using primers described previously (Powell et al. 1993; van der Luijt et al. 1994). Briefly, to investigate for mutations in exons 1–14, DNA fragments were amplified from complementary DNA templates prepared by reverse transcription (GIBCO/BRL SuperScript II RT; Grand Island, NY, USA) of messenger RNA (mRNA). Exon 15, which covers 77% of the entire coding region, was amplified directly from genomic DNA. The primers used for PCR amplification were designed to be preceded by a T7 promoter sequence and Kozak sequence for the initiation of transcription and translation (5′GGATCCTAATACGACTCACTATAGGGACCATG3′). A 25-μl reaction mixture, containing 5 μl of the PCR product, 1 μl of ^{35}S-methionine (1000 Ci/mmol; Amersham, Arlington Heights, IL, USA), 0.5 μl RNAsin (recombinant 40 U/μl), 1 μl TnT T7 RNA polymerase, 0.5 μl amino acid methionine (−) mix, and 10 μl TnT wheat germ extract (Promega, Madison, WI, USA), was incubated at 30°C for 1 h. This product (10 μl) was electrophoresed on a 4%–20% sodium dodecyl sulfate (SDS)-polyacrylamide Ready Gel (Novex, San Diego, CA, USA) for 2 h at 150 V, fixed, dried, and autoradiographed for 12–24 h, using Kodak X-Omat AR film.

Sequencing analysis

Sequencing templates were produced by PCR for samples showing aberrant mobility on SSCP or PTT. All PCR products for sequencing analysis were purified using a Qiaquick PCR purification column (Qiagen, Valencia, CA, USA) and directly sequenced using a dye terminator cycle sequencing kit and an automatic fluorescent sequencer (ABI 377; Applied BioSystems, Foster City, CA, USA) according to the manufacturer's instructions. For RT-PCR products, internal oligonucleotides were designed (sequences available on request). Sequencing reactions were carried out on both sense and antisense strands.

Results and discussion

Germline mutations of the *APC* gene were identified in 38 (61%) of the 62 Korean FAP patients. Twenty of the mutations were small deletions; 11 were point mutations resulting in stop codons; 4 were 1- to 4-base-pair insertions; 2 were sequence alterations in mRNA, and 1 was a base substitution in the splicing recognition site. Nineteen of the detected mutations were presumed to be novel.

Initially, in 48 FAP patients, we used SSCP analysis to screen for mutations as SSCP has been used widely to screen for mutations in the *APC* gene. Using this approach, we identified causative mutations (mutations resulting in truncation of the *APC* protein) in 23 patients (48%) (Fig. 1).

Because the frequency of mutations detected with SSCP was lower than expected (Miyoshi et al. 1992), and because of the observation that most of the germline *APC* gene mutations identified in FAP patients resulted in truncation of the *APC* protein, the protein truncation test (PTT) was used for 25 patients whose mutations were not identified by SSCP. Using PTT, we were able to identify mutations in an additional 6 patients. Thus, the combined use of SSCP and PTT allowed the detection of germline *APC* gene mutations in 29 of the 48 (60%) FAP patients.

In 14 newer patients, we used only PTT to screen for mutations (Fig. 1). In this group, we identified truncating mutations in 9 patients (64%). Thus, our results indicate that the mutation detection rate with PTT was superior to that with SSCP. Our overall mutation detection rate (61%) is comparable with that in a previous report (Miyoshi et al. 1992).

Table 1 shows a summary of all germline mutations and the available information on the clinical manifestations in the index case in each family (the patients in our study). All the identified mutations in the open reading frame resulted in truncations of the *APC* protein. Seventy-four % of the mutations were located in the 5′ half of exon 15. The two most common mutations seen were a 5-base pair (bp) deletion at codon 1309, in six patients (SNU-F1, F34, F42, F43, MJH-F1, and NMC-F1), and a 5-bp deletion at codon 1061 in three patients (SNU-F10, F46, and CMC-F18), which together accounted for 24% of the detected mutations. In relation to the published data, 19 of the detected mutations in our series (50%) had not been published.

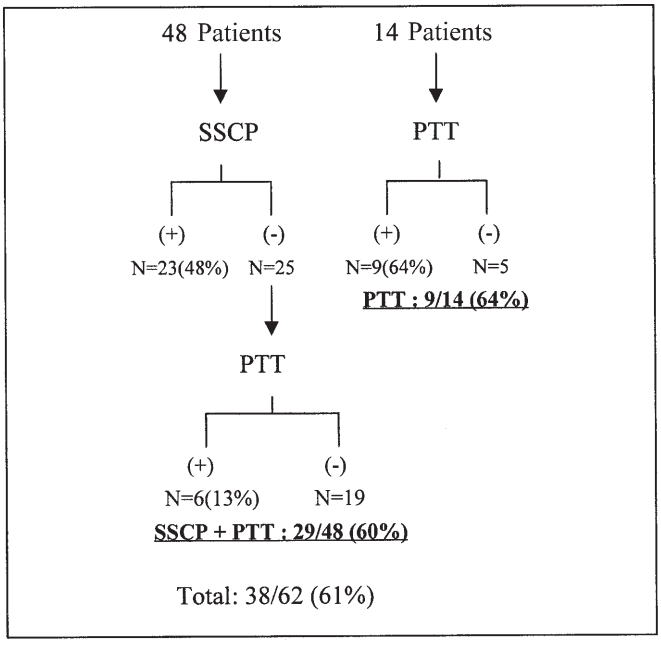

Fig. 1 Screening strategy for identification of germline mutations in the adenomatous polyposis coli (*APC*) gene in 62 unrelated familial adenomatous polyposis patients, with the results achieved by each detection method. *Arrows* indicate the flow of experiments, and the lines separate the subjects into two subgroups: those proven to have mutations by each designated screening method and those not proven to have mutations. The percentages in parentheses represent the detection rates. *SSCP*, Single-strand conformation polymorphism; *PTT*, protein truncation test; (+), mutation positive; (−), mutation negative; N, number of subjects

During this study, we also found a series of polymorphisms. Three of the polymorphisms (TAC to TAT at codon 486, GCA to GCG at codon 545, and TCG to TCT at codon 1756) had been previously reported (Miyoshi et al. 1992). Additionally, one rare polymorphism was found at the 3′ border of intron 13, 9–13 nucleotides from the 5′ end of exon 14 (AGAAA to TGTTT). The allelic frequency of that polymorphism was >0.12 in the 49 healthy unrelated persons.

In six patients (SNU-F29, CCU-F1, CNU-F4, KNH-F2, PNU-F6, and PNU-F11), the results of SSCP analysis were normal, but germline mutations were discovered by PTT. Contrary to the nucleotide deletions or insertions easily detected by SSCP, the mutations identified in these patients were single nucleotide substitutions. This finding indicates that PTT can identify additional mutations missed by SSCP alone.

In screening for mutations of the *APC* gene, many techniques, such as SSCP analysis (Groden et al. 1991) , denaturing gradient gel electrophoresis (DGGE) (Fodde et al. 1992), and ribonuclease protection assay (RPA) (Miyoshi et al. 1992), have been employed. SSCP and DGGE have been preferentially used for their simplicity. However, they are time-consuming methods for the analysis of huge genes

Table 1 Summary of germline mutations found in the adenomatous polyposis coli (*APC*) gene

Family name	Codon	Nucleotide change	Extracolonic manifestations	No. of polyps
KNH-F2[a,b]		*ag*/TT → cg/TT (splice acceptor site of exon5)	—	310
SNU-F49[c]		E6-E10 connection in mRNA *ag*/CG → cg/CG	Stomach, nonadenomatous polyps OST, DEN, ileal adenoma	More than 1000
SNU-F29[b]	216	*C*GA → TGA[d]	Stomach, nonadenomatous polyps	940
SNU-F44	428	GA*AC*C → GAC	—	More than 1000
PNU-F9	428	GA*AC*C → GAC	STO	2350
SNU-F23	487	GG*G* → GG	—	140
IJU-F3	621	AC*TTA*C → AC*CT*C	OST, CHRPE, thyroid cancer, STO Epidermoid cyst	300
SNU-F18	699	T*G*G → TAG	DEN Stomach, nonadenomatous polyps	700
SNU-F35	740	GATGC → GATGC*ATGC*	—	130
SNU-F4	772	*A*CT → CT	DEN, CHRPE Stomach, nonadenomatous polyps	450
SNU-F41	849	GA*TA*G → GAG[d]	Desmoid tumor	1250
SNU-F48	864	CAT → CAT*CT*	OST, CHRPE, epidermal cyst	3000
CCU-F1[b]	932	T*C*A → TGA	—	250
PNU-F6[b]	935	TA*C* → TAA[d]	Thyroid cancer	760
PNU-F11[b]	935	TA*C* → TAA	—	Unknown
HYU-F14	936	TACA → TAC*C*A	STO	400
SNU-F19	997	TAT → TA*A*T	CHRPE, epidermoid cyst	450
HYU-F11	999	*C*AA → TAA	—	790
SNU-F10	1061	AA*ACAAA*G → AAG[d]	—	2000
CMC-F18	1061	AA*ACAAA*G → AAG	Desmoid tumor	1000
SNU-F46	1061	AA*ACAAA*G → AAG	Thyroid cancer	250
SNU-F30	1075	TA*T* → TAA	CHRPE	More than 1000
KSU-F2	1075	TA*T* → TAA	Unknown	Unknown
SNU-F14	1098	TG*TG*T → TGT	CHRPE	Numerous
SNU-F37	1098	TG*TG*T → TGT	DEN, CHRPE	1400
SNU-F13	1185	*ACAGA*TAT → TAT[d]	—	Numerous
YSU-F6	1211	*AT*G → G	—	150
SNU-F25	1256	*C*AA → TAA	STO, DUO, ileal adenoma	1400
YSU-F5	1289	TG*T* → TGA	—	500
SNU-F1	1309	GA*AAAGA*T → GAT[d]	DEN, DUO, OST, CHRPE Desmoid tumor	8000
SNU-F34	1309	GA*AAAGA*T → GAT	—	1000
SNU-F42	1309	GA*AAAGA*T → GAT	—	1300
SNU-F43	1309	GA*AAAGA*T → GAT	CHRPE, STO, thyroid cancer	750
MJH-F1	1309	GA*AAAGA*T → GAT	DUO, ileal adenoma	More than 1000
NMC-F1	1309	GA*AAAGA*T → GAT	Epidermoid cyst	More than 1000
CNU-F4[b]	1370	*A*AA → TAA	DUO, desmoid tumor	2000
JIH-F1	2040	*C*AG → TAG	STO CA	Unknown

OST, Osteoma; DEN, dental anomaly; CHRPE, congenital hypertrophy of the retinal pigment epithelium; STO, stomach adenoma; STO CA, stomach cancer; DUO, duodenal adenoma

[a] A 16-base pair (bp) nucleotide deletion in the cDNA was detected with the protein truncation test (PTT) and direct sequencing of the cDNA. The exon boundary is indicated by the slashed line. This mutation leads to a frameshift and a stop codon 8 bp downstream in the new reading frame

[b] The mutation was not identified by single-strand conformation polymorphism analysis, but the abnormal protein was observed in the PTT

[c] Exon skipping (exons 7, 8, and 9) was identified by the PTT and direct sequencing of the cDNA. In this patient, exon 6 was directly connected to exon 10, which leads to a frame shift

[d] The same mutations have previously been reported in the literature

such as *APC* because they are capable of screening only small pieces of DNA, due to the limitation of DNA-size resolution on gel (Sheffield et al. 1993). Also, as the size of the DNA increases, generally the sensitivity falls. The optimal size of DNA fragments for sensitive base substitution detection by SSCP is 200 bp. Therefore, the failure of SSCP to detect some mutations could be due to inherent defects in SSCP or possibly, could result from poor gel running conditions. Consequently, SSCP is not a convenient method for the analysis of mutations in large, multi-exonic genes such as *APC*.

Our results indicate that it would be more practical to employ PTT alone for rapid and accurate screening for *APC* gene mutations in FAP patients. Since 76% of the detected mutations in our series were located between codons 699 and 1370, and 67% of the reported mutations are located between codons 680 and 1700, it would be logical to perform PTT of this region first, using two PTT sets covering codons 680–1700.

The two most common mutations we observed were a 5-base pair (bp) deletion at codon 1309 in six patients and a 5-bp deletion at codon 1061 in three patients, which together accounted for 24% of the detected mutations. These two codons have been reported to be the most frequent sites of mutation (15%–26%) by others (Cottrell et al. 1992; Nagase et al. 1992; Miyoshi et al. 1992; Ando et al. 1993; Groden

et al. 1993; Varesco et al. 1993; Caspari et al. 1994; van der Luijt et al. 1997).

In 24 patients (39%), our approach did not reveal the underlying genetic defect. This presents a major problem for the presymptomatic diagnosis of FAP by mutation analysis. The most likely explanations for the failure to identify mutations in these patients include promoter or enhancer site mutations, gross structural rearrangements, or the existence of another candidate gene for FAP (Kraus et al. 1994; McPherson et al. 1994; van der Luijt et al. 1997).

APC gene mutations at different positions have been reported to account for some of the differences in the phenotypic manifestations of FAP (Gayther et al. 1994; Wallis et al. 1994; Caspari et al. 1995; Dobbie et al. 1996). Patients with mutations between codons 1444 and 1578 were almost invariably found to develop desmoid tumors (Caspari et al. 1995). In our series, however, there was no patient with a mutation in this region of the *APC* gene, and four patients who developed desmoid tumors had mutations outside this region.

It has also been reported that a germline mutation in codon 1309 of the *APC* gene leads to the development of colonic polyps at a younger age, giving rise to early-onset colorectal cancer (Nagase et al. 1992; Caspari et al. 1994; Gayther et al. 1994). In our series, the mean age at the time of diagnosis of FAP was 29 years in the six patients with mutations in codon 1309, somewhat younger than the mean age of 36 years in patients whose mutations were located elsewhere in the *APC* gene.

Mutations at the 5′ end of *APC* are associated with relatively few colonic polyps, usually fewer than 100, and with a later onset of symptoms, but still a significant risk of colon cancer (Spirio et al. 1993). This condition is referred to as attenuated adenomatous polyposis coli (AAPC). Our patient KNH-F2 had the greatest 5′ end mutation, a splice acceptor site mutation at exon 5, which is beyond the functional boundary of AAPC at codon 168 (Olschwang et al. 1993).

Congenital hypertrophy of the retinal pigment epithelium (CHRPE), a retinal lesion frequently associated with FAP, is associated with mutations located between codons 457 and 1445 of the *APC* gene (Wallis et al. 1994; Caspari et al. 1995). In eight patients who had CHRPE in our series, all the identified mutations were located within this boundary.

Identification of the *APC* mutations responsible for FAP in our series of families should have a great impact on management. The other family members at risk can be easily diagnosed within 1 or 2 days (Park et al. 1994). Subsequently, family members can be divided into those carrying the mutated *APC* genes (who can benefit from appropriate management to prevent colorectal cancer) and those without the mutations (who can be relieved of the anxiety of developing the disease).

Our results in this study confirmed that the PTT was superior to SSCP for the detection of germline mutations of the *APC* gene responsible for FAP. The employment of PTT would facilitate the detection of germline mutations of the *APC* gene and aid in the management of families affected with FAP.

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