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Three novel *APC* mutations in Thai familial Adenomatous Polyposis patients

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ABSTRACT

Familial adenomatous polyposis (FAP) is an autosomal dominant genetic disease that is characterized by multiple adenomatous polyps in the colon and rectum. Adenomatous polyposis coli gene (*APC*) mutations are the causative event for FAP. *APC* consists of 15 exons, which encode 2,843 amino acids. The APC protein acts as a tumor suppressor and plays a role in several cellular processes. Here we investigated *APC* mutations in 20 unrelated Thai patients with FAP using denaturing high-performance liquid chromatography followed by DNA sequencing. Twenty mutations were identified in this study. Three mutations were novel mutations, including one nonsense mutation (c.2560A>T), one deletion mutation (c.4634_4638delCAAAT) and one indel mutation (c.3746_3748delinsAA). Data from mutations in Thai patients with FAP will be useful for molecular genetics diagnosis and genetic counseling of patients with FAP.

Keywords: familial adenomatous polyposis; *APC* gene; DHPLC; mutations

INTRODUCTION

Familial adenomatous polyposis (FAP) is an autosomal dominantly inherited disorder with an incidence of approximately 1/10,000 (Bulow *et al*., 1996; Plawski *et al*., 2004). FAP is characterized by the presence of hundreds to thousands of adenomatous polyps in the colon and rectum. Polyps occur in the second decade of life and will develop to colorectal cancer in the third or fourth decade of life if the polyps are not removed by colectomy (Bisgaard *et al*., 1994). In classical FAP, at least 100 polyps are present, while polyposis with fewer than 100 polyps per patient is referred to as attenuated polyposis (AFAP). The development of extra-colonic manifestations, such as congenital hypertrophy of the retinal pigment epithelium (CHRPE), desmoid tumors and duodenal adenomas, are also found in FAP patients (Nieuwenhuis and Vasen, 2007).

FAP is caused by a germline mutation in the adenomatous polyposis coli (*APC*) gene. *APC* is located on chromosome 5q21 and encodes a 312-kDa protein consisting of 2,843 amino acids (Groden *et al*., 1991; Kinzler *et al*., 1991). APC has an important role in the regulation of β-catenin that involves the Wnt signaling pathway. APC forms a complex with β-catenin, axin and GSK3β, which regulates the amount of β-catenin for transcriptional activation (Nathke, 2004). When *APC* is mutated, β-catenin accumulates in the cytoplasm, binds to transcription factors, and alters the expression of various genes affecting the proliferation, differentiation and apoptosis of cells. Therefore, inactivation of the *APC* gene product constitutes the initial step in the development of abnormal growth of polyps. *APC* is expressed in a variety of fetal and adult tissues, including mammary and colorectal epithelium (Nishisho *et al*., 1991). The *APC* gene is constitutively mutated in up to 95% patients who are affected with FAP (Beroud and Soussi, 1996; Laken *et al*., 1999). More than 5,500 mutations of *APC* have been identified (CNIL, 2014). The most common germline mutations involve the introduction of a premature stop codon, either by a nonsense mutation, frameshift mutation or missense mutation, which leads to truncation of the protein in the C-terminal region. The hot spot mutations of *APC* are located at positions 1062 and 1309 (Nagase and Nakamura, 1993). In Thai population, the mutation was found in exon 4 (c.481C>T) in family with attenuated

familial adenomatous polyposis (AFAP) (Poovorawan *et al*., 2012). However, data on *APC* mutations in Thai patients are still limited, and collecting more data of *APC* mutations from different populations worldwide will be useful to better our understanding of its role in FAP. Therefore, the aim of this study was to investigate the types of *APC* gene mutations in Thai FAP patients. These data will be useful for the development of molecular diagnostic tools for FAP.

MATERIALS AND METHODS

Subjects and DNA extraction

Twenty unrelated Thai patients with FAP from the Department of Medicine, Faculty of Medicine Siriraj Hospital, Mahidol University, Thailand, were included in this study. The patients were diagnosed after clinical examinations showing multiple adenomatous polyps forming in the colon and rectum at puberty, but rarely before puberty. The healthy volunteers who had no clinical symptom of FAP and no family history of FAP were selected to be a control group. All patients gave written informed consent for this study. The study was approved by the Institutional Review Board (COA no.Si181/2014). Blood samples were collected in EDTA-containing tubes. A standard phenol-chloroform method was used for genomic DNA preparation (Laird *et al*., 1991).

APC-DNA amplification

The entire coding sequence of the *APC* gene (NM_001127511) was amplified by PCR using the primers listed in Table 1. The PCR reaction mixture contained 5 µl of detergent-free 5× Phusion HF reaction buffer containing 7.5 mM $MgCl_2$, which provides 1.5 mM $MgCl_2$ in the final reaction, 1 µl of 10 pmol forward and reverse primers, 0.5 µl of 10 mM deoxynucleotide triphosphate (dNTP) mixture, 0–4 µl of 50 mM $MgCl_2$ (0-8 mM $MgCl_2$ at final concentration), 4 µl of 20 ng/µl DNA template (80 ng of DNA in reaction), 0.1 µl of 2 U/µl Phusion enzyme (0.008U per reaction) (Phusion, Finnzyme, Finland) and deionized water to a total reaction volume of 25 µl. PCR amplifications were performed using a Thermal Cycler (Perkin Elmer PCR 2400, USA) under the following conditions: after initial denaturation at 98°C for 30 s, 35 cycles of 98°C denaturation for 10 s, 20 s annealing (annealing temperatures are shown in Table 1) and 72°C extension for 30 s were carried out, followed by a final extension step at 72°C for 5 min. Aliquots (5 µl) of PCR products were loaded onto 1.5% agarose gels to verify the size and quantity of products.

Mutation screening by denaturing high-performance liquid chromatography (DHPLC) analysis

The PCR products were denatured at 95°C for 5 min and re-annealed by gradual decrease of temperature at a rate of 1°C per 22 s until reaching 25°C using the thermal cycler to generate the elution profile before injection. An aliquot of 5–8 µl of each re-annealed PCR product was directly injected into a DNASep HT column (Transgenomic Inc., Omaha, NE, USA). Data were analyzed by Navigator™ software (Transgenomic Inc.).

DNA sequencing

The mutation fragments analyzed by DHPLC that showed abnormal peaks were purified using the QIAquick PCR Purification Kit (QIAGEN, Hilden, Germany) or Exo-SAP-IT (USB Corporation, Cleveland, OH, USA) according to the manufacturer's protocol, and were subjected to DNA sequencing using the DYEnamic™ ET Dye Terminator Kit (Amersham, UK) and MegaBACE™ 1000 Automated DNA sequencer (Amersham Life Science Inc., Cleveland, OH, USA).

RESULTS

DHPLC was performed to analyze all 31 fragments of PCR products from 20 unrelated FAP patients. Three novel mutations were found including one nonsense mutation (c.2560 A>T), one deletion mutation (c.4634_4638delCAAAT) and one indel mutation (c.3746_3748delinsAA) (CNIL, 2014). The DNA sequencing of three novel mutations showed in Figures 1–3. Mutations in *APC* were identified in the fragment 4F/4R in one patient, fragment 9F/9R in one patient, fragment 14F/14R in one patient and fragment 15F/15R in 17 patients (Table 2). Nonsense mutations were detected in 9 patients, including c.1297C>T, c.1787C>G, c.2560A>T, c.2805C>G, c.3000C>A, c.3103C>T, c.3225T>G, c.3827C>G and c.4067C>G. The substitution mutations resulted in substitution with a stop codon (TAA, TAG and TGA). Eight deletion mutations were detected in 10 patients, including deletion of 5 nucleotides (c.3183_3187delACAAA, c.3927_3931delAAAGA and c.4634_4638delCAAAT) and deletion of two nucleotides (c.449_450delAA, c.2396_2397delAT, c.3086_3087delTT, c.4219_4220delAG and c.4393_4394delAG). An indel mutation was found in one patient (c.3746_3748delinsAA). Each deletion or indel resulted in an unrecognized amino acid sequence after the mutated codon and eventually created a premature stop codon.

Table 1 Primers for PCR amplification of the *APC* gene.

Primers	Nucleotide sequence	Product size (bp)	Position	$MgCl_2$ (mM)	PCR Conditions			No. of cycles
					Denature	Annealing	Extension	
1F	5′- TTTCTTTAAAAACAAGCAGCCA -3′	285	16919-17203	1.5	98°C 10 s	59°C 20 s	72°C 30 s	35
1R	5′- GTTTACAAGAGGGAATACTG -3′							
2F	5′- GCGTGCTTTGAGAGTGATCTGA -3′	303	28322-28624	1.5	98°C 10 s	56°C 20 s	72°C 30 s	35
2R	5′- ACCAACACCCAAATCGAGAG -3′							
3F	5′- TTAAGAATATTTTAGACTGCTT -3′	316	29240-29555	4	98°C 10 s	56°C 20 s	72°C 30 s	35
3R	5′- ACAATAAACTGGAGTACACAAGGC -3′							
4F	5′- TGCTCTTCTGCAGTCTTTATTA -3′	228	37680-37907	2	98°C 10 s	50°C 20 s	72°C 30 s	35
4R	5′- CTTTAAAATATCAAGTTATTA -3′							
5F	5′- TTTACTGATTAACGTAAATACA -3′	225	42847-43071	1.5	98°C 10 s	50°C 20 s	72°C 30 s	35
5R	5′- ATTTTATTCCTAATAGCTCT -3′							
6F	5′- GCCATAGTATGATTATTTCTAT -3′	243	54488-54730	1.5	98°C 10 s	55°C 20 s	72°C 30 s	35
6R	5′- TGGTGTCACATTAAATTATACA -3′							
7F	5′- AAGAAAGCCTACACCATTTTT -3′	238	63341-63578	1.5	98°C 10 s	56°C 20 s	72°C 30 s	35
7R	5′- GATCATTCTTAGAACCATCTTGC -3′							
8F	5′- ATGTTATCTGTATTTACCTAT -3′	221	77549-77769	1.5	98°C 10 s	52°C 20 s	72°C 30 s	35
8R	5′- CCAAGAATGTCTTAGCAA -3′							
9F	5′- TGGTTTTTGGCTTTTGGATA -3′	484	81019-81502	1.5	98°C 10 s	60°C 20 s	72°C 30 s	35
9R	5′- TTGCTTTGAAACATGCACTAC -3′							
10F	5′- AAACATCATTGCTCTTCAAATAACAA -3′	217	83951-84167	1.5	98°C 10 s	53°C 20 s	72°C 30 s	35
10R	5′- CTACCATGATTTAAAAATCCACCAGT -3′							

Table 1 continued.

Primers	Nucleotide sequence	Product size (bp)	Position	$MgCl_2$ (mM)	PCR Conditions			No. of cycles
11F	5′- CTAGTATTTAAGTTACCAACT -3′	281	89141-89421	1.5	98°C 10 s	52°C 20 s	72°C 30 s	35
11R	5′- ATGAAAGTAAATTAACTCATA -3′							
12F	5′- AGCTTGGCTTCAAGTTGTCTT -3′	195	89988-90182	1.5	98°C 10 s	62°C 20 s	72°C 30 s	35
12R	5′- AGTGAGACCCTGCCTCAAAG -3′							
13F	5′- TTTCTATTCTTACTGCTAGCATTAAAAACA -3′	307	90883-91189	1.5	98°C 10 s	53°C 20 s	72°C 30 s	35
13R	5′- AATACACAGGTAAGAAATTAGGAAATCTCAT -3′							
14F	5′- TGAGAGACAAATTCCAACTCTA -3′	368	97012-97379	2	98°C 10 s	50°C 20 s	72°C 30 s	35
14R	5′- GCTTAAAACTTTCATGATTAT -3′							
15.1F	5′- ATGCCTTTTGTCTTCTATCCT -3′	473	99582-100055	1.5	98°C 10 s	55°C 20 s	72°C 30 s	35
15.1R	5′- CCTTGGGACTTAAATTGTCTAT -3′							
15.2F	5′- CCCTAGAAGCAGAATTAGATG -3′	548	99984-100531	1.5	98°C 10 s	55°C 20 s	72°C 30 s	35
15.2R	5′- TTCCGACTTAGTGAAATTGTAA -3′							
15.3F	5′- GAAGACAGAAGTTCTGGGTCTAC -3′	419	100412-100829	1.5	98°C 10 s	62°C 20 s	72°C 30 s	35
15.3R	5′- GCCTTCCAGAGTTCAACTGC -3′							
15.4F	5′- CCTAGCCCATAAAATACATAGT -3′	520	100717-101236	1.5	98°C 10 s	55°C 20 s	72°C 30 s	35
15.4R	5′- AGGCTGATCCACATGACGTTTC -3′							
15.5F	5′- CATGAAGAAAGAAGAGAGACCAA -3′	538	101165-101702	3	98°C 10 s	62°C 20 s	72°C 30 s	35
15.5R	5′- TTCTAGGGTGCTGTGACACTG -3′							
15.6F	5′- CAGACGACACAGGAAGCAGA -3′	542	101579-102120	1.5	98°C 10 s	62°C 20 s	72°C 30 s	35
15.6R	5′- GCAGCTTGCTTAGGTCCACT -3′							

Table 1 continued.

Primers	Nucleotide sequence	Product size (bp)	Position	$MgCl_2$ (mM)	PCR Conditions			No. of cycles
15.7F	5′- AAACCAAGCGAGAAGTACCTAA -3′	481	102048-102528	1.5	98°C 10 s	62°C 20 s	72°C 30 s	35
15.7R	5′- CTTGCCACAGGTGGAGGTAAT -3′							
15.8F	5′- ATGCCAACAAAGTCATCACGTA -3′	556	102455-103010	1.5	98°C 10 s	54°C 20 s	72°C 30 s	35
15.8R	5′- TACCATCTAACTGATTTTTG -3′							
15.9F	5′- AAGGGAAAAGTCACAAGC C -3′	458	102909-103366	1.5	98°C 10 s	54°C 20 s	72°C 30 s	35
15.9R	5′- TTCTTTTGCCTTTCTTAATTCA -3′							
15.10F	5′- CTTACTGTTTTTCACGAAATGA -3′	482	103266-103747	1.5	98°C 10 s	57°C 20 s	72°C 30 s	35
15.10R	5′- ATGAAATGATTTAGGAGCATA -3′							
15.11F	5′- AGAGACTGAGCCCCCTGACTCA -3′	513	103672-104184	1.5	98°C 10 s	62°C 20 s	72°C 30 s	35
15.11R	5′- AAGGGTTTTCTTCTTGATCAG -3′							
15.12F	5′- GCTGCTGCTGCATGTTTATCTA -3′	452	104063-104514	1.5	98°C 10 s	62°C 20 s	72°C 30 s	35
15.12R	5′- GTGGTGGCTGTTTGACCTTC -3′							
15.13F	5′- TTCGAAATAGCTCCTCAAGTAC -3′	458	104415-104872	1.5	98°C 10 s	56°C 20 s	72°C 30 s	35
15.13R	5′- GGCATTCTTGGATAAACCT -3′							
15.14F	5′- GTCATATACATCTCCAGGTA -3′	516	104800-105315	1.5	98°C 10 s	54°C 20 s	72°C 30 s	35
15.14R	5′- GGACTTTCAGAATGAGACCG -3′							
15.15F	5′- CACCTAATCTCAGTCCCACTAT -3′	537	105231-105767	1.5	98°C 10 s	56°C 20 s	72°C 30 s	35
15.15R	5′- AAACACTGTCAATCACCG -3′							
15.16F	5′- GGTGCTGAATCAAAGACTCTAA -3′	423	105617-106039	1.5	98°C 10 s	56°C 20 s	72°C 30 s	35
15.16R	5′- AACAGTCCCACTAGGTGAA -3′							
15.17F	5′- CCCTGTATCAGAGACTAATGAA -3′	432	105946-106377	1.5	98°C 10 s	56°C 20 s	72°C 30 s	35
15.17R	5’-CCAGAACAAAAACCCTCTAA -3’							

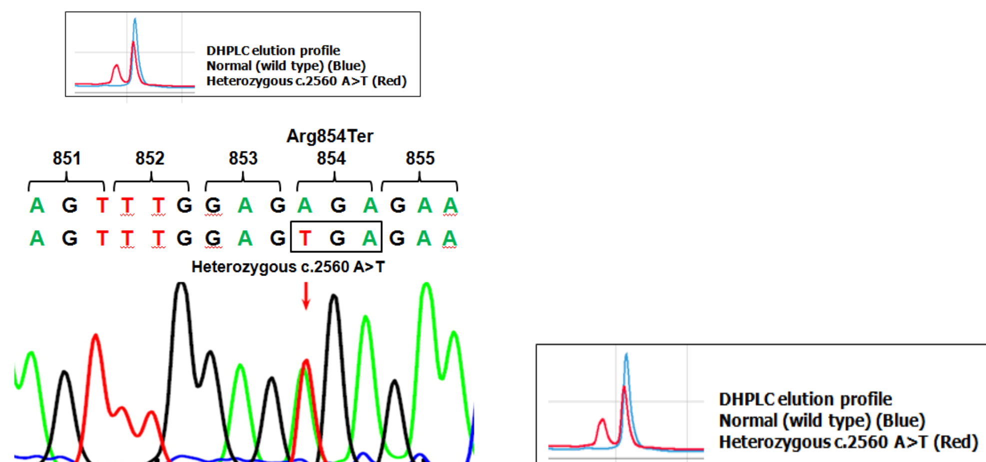

Figure 1 Heteroduplex profile from DHPLC analysis and DNA sequencing show heterozygous c.2560 A>T in FAP02 patient.

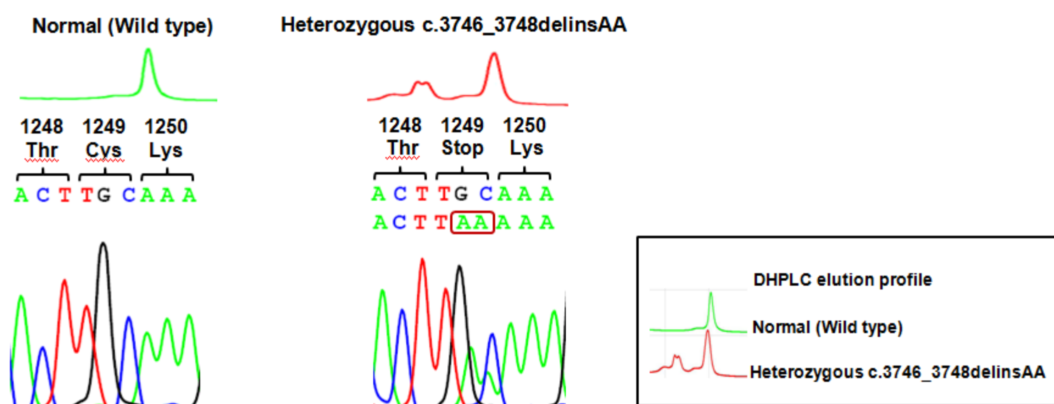

Figure 2 Heteroduplex profile from DHPLC analysis and DNA sequencing show heterozygous c.3746_3748delinsAA in FAP06 patient.

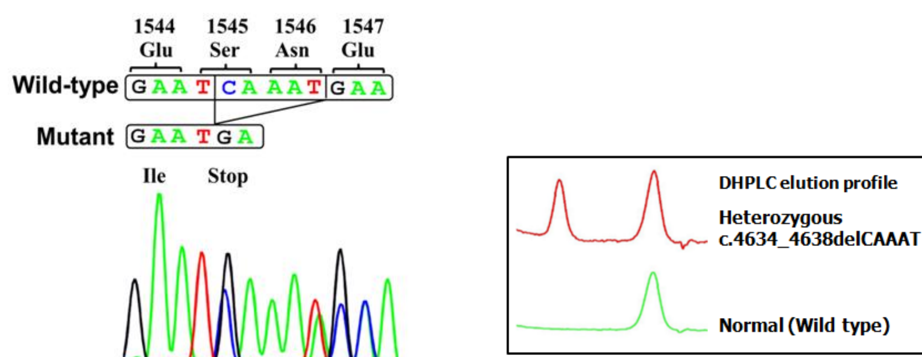

Figure 3 Heteroduplex profile from DHPLC analysis and DNA sequencing show heterozygous c.4634_4638delCAAAT in FAP18 patient.

Segregation analysis of mutation within FAP06 patient's family was performed by direct detection mutation of c.3746_3748delinsAA using DHPLC and DNA sequencing. The result showed the presence of c.3746_3748delinsAA in two out of four members of family (Figure 4). In the FAP06 family, III-1 was a proband who carried one allele of c.3746_3748delinsAA that generated a stop codon of APC protein. A sample from the sister of the proband (III-3) showed the c.3746_3748delinsAA and found the polyps in this member that same as the proband. The family history of this family revealed that the cancer affected members in every generation as a pattern of autosomal dominant inheritance in the family.

Table 2 Clinical manifestation and *APC* genotype of Thai patients with FAP.

Patient	Sex	FAP/AFAP	Extra-colonic manifestations	Exon	Nucleotide change (codon change)
FAP01	F	AFAP	CHRPE	15	c.3000C>A (p.Tyr1000Ter)
FAP02	M	FAP (Profuse polyps)	-	15	c.2560A>T (Arg854Ter) [a]
FAP03	F	FAP (Profuse polyps)	-	15	c.3086_3087delTT (p.Leu1029Glnfs*5)
FAP04	F	FAP (Profuse polyps)	CHRPE, Duodenal polyps	15	c.4067C>G (p.Ser1356Ter)
FAP05	F	FAP	CHRPE, Duodenal and stomach polyps and Desmoid fibromatosis	15	c.3927_3931delAAAGA (p.Glu1309Aspfs*4)
FAP06	F	FAP	-	15	c.3746_3748delinsAA (p.Cys1249Ter) [a]
FAP07	F	FAP	-	9	c.1297C>T (p.Gln433Ter)
FAP08	F	FAP	Fibromatosis at back	15	c.2396_2397delAT (p.Tyr799Cysfs*3)
FAP09	F	FAP	-	15	c.3183_3187delACAAA (p.Gln1062Terfs*1)
FAP10	M	FAP	-	15	c.3827C>G (p.Ser1276Ter)
FAP11	M	FAP	-	4	c.449_450delAA (p.Lys150Argfs*17)
FAP12	M	FAP	-	15	c.3103C>T (p.Gln1035Ter)
FAP13	M	FAP	-	15	c.4219_4220delAG (p.Ser1407Terfs*1)
FAP14	M	FAP	-	15	c.2805C>G (p.Tyr935Ter)
FAP15	M	FAP	-	15	c.3927_3931delAAAGA (p.Glu1309Aspfs*4)
FAP16	F	FAP	-	15	c.3225T>G (p.Tyr1075Ter)
FAP17	F	FAP	Desmoid fibromatosis	15	c.4393_4394delAG (p.Ser1465Trpfs*3)
FAP18	F	FAP	Soft tissue tumor at back, Recurrent mass at breast	15	c.4634_4638delCAAAT (p.Ser1545Terfs*1) [a]
FAP19	M	AFAP	CHRPE	14	c.1787C>G (p.Ser596Ter)
FAP20	F	FAP	CHRPE, Desmoid fibromatosis	15	c.3183_3187delACAAA (p.Gln1062Terfs*1)

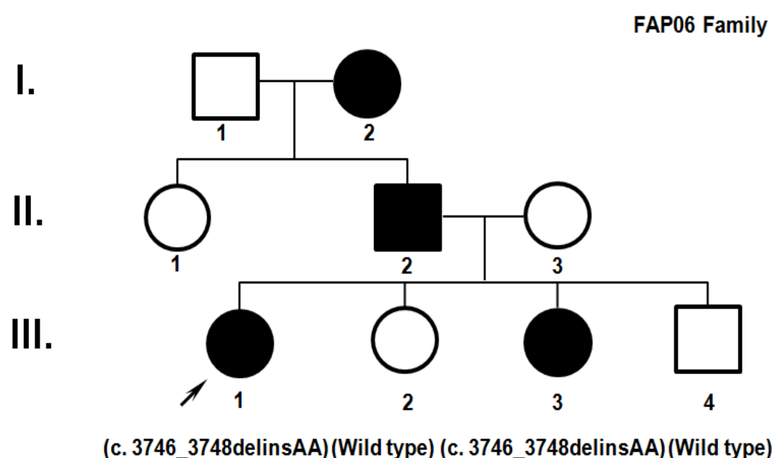

Figure 4 Pedigree of FAP06 family. Mutation c.3746_3748delinsAA was detected in the proband (III-1) and member III-3.

DISCUSSION

Mutations in the *APC* gene have been studied for several decades by many research groups worldwide and more than 5,500 mutations have been identified (CNIL, 2014). In this study, we identified APC mutations in 20 unrelated FAP patients as summarized in Table 2. Mutations were located in exons 4, 9, 14 and 15, and the mutations were distributed most frequently in exon 15 (85%). Three of the mutations, including one nonsense (c.2560A>T), one deletion mutation (c.4634_4638delCAAAT) and one indel mutation (c.3746_3748delinsAA), were novel mutations

The novel mutations c.2560 A>T and c.3746_3748delinsAA created a stop codon at position 854 and 1249, respectively. Mutations in these regions resulted in protein truncation, which can lead to accumulation of *β-catenin* in the cytoplasm, binding to transcription factors and altered expression of various genes affecting proliferation, differentiation, migration and apoptosis (Sparks *et al.*, 1998). The regulation of beta-catenin signaling is controlled by the B56 subunit of protein phosphatase 2A. These novel mutations in APC may affect the degradation of *β-catenin* thereby retaining *β-catenin* in the cells (Seeling *et al.*, 1999). The novel mutation c.4634_4638delCAAAT causes a frameshift translation beginning at codon 1545 that results in removal of the SAMP repeat. These mutations may inactivate APC and cause *β-catenin* to accumulate, resulting in activation of *β-catenin*/TCF-mediated transcription, which is similar to the predicted results of the mentioned nonsense mutations (Sparks *et al.*, 1998).

The c.3183_3187delACAAA mutation is located in exon 15 of the *APC* gene and causes a frameshift translation beginning at codon 1062. This results in protein truncation of a 20 amino acid repeat domain through the C-terminus. The predicted phenotypes of the patient were correlated with the clinical diagnosis, which are profuse polyps and CHRPE. The c.3927_3913delAAAGA mutation is located in exon 15 of the *APC* gene and causes a frameshift translation beginning at codon 1309, introducing three novel amino acids and a premature termination at codon 1312. This protein truncation begins at the 20 amino acid repeat domain through the C-terminus. Therefore, the predicted phenotypes are severe as mentioned above and correlate to the clinical diagnosis. The c.3183_3187delACAAA and c.3927_3913delAAAGA mutations are hot spot germline mutations in *APC*. We identified c.3183_3187delACAAA and c.3927_3913delAAAGA mutations in two patients (10%) each. In other populations, the 5-bp deletion at codon 1062 also occurred with a range of frequencies varying from 0% in northwestern Spanish and 1.5% in Israeli to 8.4% in Australians (Gavert et al., 2002; Ruiz-Ponte et al., 2001; Schnitzler et al., 1998; Varesco et al., 1993). The frequency of c.3927_3913delAAAGA mutation varies from 0% in northwestern Spanish, 2.4% in Australian, 5% in Dutch, 7% in Israeli to 16% in Italians (Gavert *et al.*, 2002; Ruiz-Ponte *et al.*, 2001; Schnitzler *et al.*, 1998; Varesco *et al.*, 1993).

The particular manifestations of FAP are frequently correlated with the position of *APC* mutations. Classical FAP is found in patients with mutations between codons 168 and 1580 of *APC*, while patients with mutations in codons 1250–1464 are generally characterized by severe FAP (Half *et al.*, 2009). The mutations in AFAP patients are located before codon 157 and after codon 1580 (Nieuwenhuis

and Vasen, 2007), while the mutation at codon 161 was found in Thai patients with AFAP (Poovorawan *et al*., 2012). In this study, most patients with classical FAP showed mutations between codons 433–1545. However, patients with AFAP harbored mutations at codons 596 and 1000, which differs from previous reports (Half *et al*., 2009; Nieuwenhuis and Vasen, 2007; Poovorawan *et al*., 2012). CHRPE is commonly associated with FAP patients who have mutations in codons 450–1444 (Polakis, 1997), and our findings are consistent with this study. The FAP patients with CHRPE showed mutations between codons 596–1356.

Results from mutation analysis shown in this study should be helpful in early detection of APC mutations in FAP family members who do not show any clinical manifestation and may help for selection of FAP family members for genetic counseling prior to genetic screening.

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