Genetic Analysis of the APC Gene in Taiwanese Familial Adenomatous Polyposis

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Key Words

Denaturing high-performance liquid chromatography · Sequence · APC · Taiwan · Familial adenomatous polyposis

Abstract

Colorectal cancer has become the third leading cause of death from cancer in Taiwan. Familial adenomatous polyposis (FAP) is an autosomal dominant inherited disease characterized by the presence of multiple adenomatous polyps in the colon and rectum. The gene responsible for FAP (*APC*) was cloned in 1991. Extensive analyses of the mutation spectra in FAP kindreds have been performed in different countries, but the results have been highly variable (30–80%). In this study, we used denaturing high-performance liquid chromatography (DHPLC) followed by automatic sequencing in an effort to establish the mutation spectrum of *APC* from DNA of peripheral blood cells. Among the 6 FAP probands analyzed, mutations were detected in 3 (50%), 2 of which were novel. The first novel mutation was at codon 2166, with a C to T transition, resulting in a stop codon. The second novel mutation was at codon 1971, with a C to G transversion, resulting in an amino acid change from serine to cysteine. The third mutation involved an A insertion in the sequence of -AAAAA- at codons 1554–1556, which created a downstream stop codon (codon 1558). This

study is the first to report mutation analysis in Taiwanese FAP probands.

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Introduction

Colorectal cancer is one of the most common forms of neoplasm and the incidence is increasing in industrial countries [8]. Although currently colorectal cancer is not the leading cause of cancer mortality in Taiwan, it is noteworthy that the incidence of colorectal cancer has increased recently [14]. Analysis of the leading causes of death from cancer between 1987 and 2001 showed that colorectal cancer has become the third leading cause of cancer mortality in Taiwan. The annual mortality from this disease increased from 1,400 to 3,457 during the same period. Thus, colorectal cancer has become one of the most important diseases in Taiwan and is likely to continue to increase in prevalence.

In the past decade, a number of important discoveries have helped to elucidate the molecular mechanisms of tumorigenesis of colorectal cancers. These include findings regarding the involvement of activation of specific oncogenes, inactivation of tumor suppresser genes and inactivation of DNA repair systems. Familial adenomatous polyposis (FAP) and hereditary non-polyposis colorectal cancer are two well-characterized hereditary colo-

rectal cancers. FAP is an autosomal dominant inherited disease characterized by the presence of multiple adenomatous polyps in the colon and rectum. Affected individuals may develop as many as thousands of adenomatous polyps in the colon. The first polyps occur, on average, at age 15 [27, 28]. If left untreated, cancer inevitably develops at a median age of 39 years [2]. FAP is also associated with extracolonic manifestations including desmoids, osteomas, epidermoid cysts, congenital hypertrophy of the retinal pigment epithelium and other tumors (brain, thyroid and liver) [11, 24]. APC, the gene responsible for FAP, was cloned in 1991 and was found to be located at chromosome 5q [3, 12, 18]. The APC gene is a negative regulator of β -catenin and is considered to play a role as a gatekeeper in the adenoma-carcinoma sequence [1].

A small proportion of FAP patients have a cytogenetically visible interstitial deletion of the long arm of chromosome 5, while some of these patients have the entire APC gene removed [15, 21]. However, the vast majority of FAP patients bear a genetic alteration involving mutations in either a single base pair or a small number of base pairs. Over 600 different germ line mutations in APC have been documented using different techniques [11, 31]. Subsequently, mutation spectra in FAP kindreds of different countries were established and showed highly variable results (30–80%), suggesting variation among different ethnic groups [5, 6, 11, 17, 19, 24, 25, 29].

Thorough understanding of the molecular mechanisms will help to classify cases of hereditary colorectal cancer and to design optimal therapies. Individuals from families with a high risk of colorectal cancer (hereditary non-polyposis colorectal cancer and FAP) should be offered genetic counseling, predictive molecular testing and, when indicated, endoscopic surveillance at appropriate intervals [13]. Likewise, chemoprevention can be prescribed for those with a diagnosis of hereditary colorectal cancer. Clinical surveillance and chemoprevention can be restricted to patients with germ line defects. In the present study, we conducted a mutation analysis of the APC gene in Taiwanese FAP kindreds in an effort to facilitate the possibility of taking such clinical measures.

Patients and Methods

Patients

This study included 6 probands from unrelated families registered at the National Taiwan University Hospital or Buddhist Tzu Chi Hualien General Hospital. Patients were referred to this study initially based on clinically suspicious colonoscopic findings, and the diagnosis of hereditary colon cancer was subsequently confirmed by surgery and/or suggestive family history of FAP, multiple polyps or

colon cancer. An initial screening was performed on a single proband from each of the 6 unrelated families. Additional family members, when available, were tested for the segregation of specific molecular variants. After completion of a thorough genetic counseling session and signing of the informed consent form for participation in the study, a 20-ml blood sample was drawn in EDTA from each individual for the preparation of genomic DNA and RNA samples. To further clarify the identified changes as a polymorphism or mutation, population analysis with genomic DNA of 250 anonymous individuals from the DNA bank of the Department of Medical Genetics of the National Taiwan University Hospital was used as the population control.

DNA Purification

DNA was purified from 200 μ l of whole blood using the Viogene blood and tissue genomic DNA extraction kit (Viogene, Taipei, Taiwan) according to the manufacturer's instructions.

PCR Conditions

Polymerase chain reactions of APC exons were performed in a 50- μ l volume containing 10 mmol/l Tris-HCl, pH 8.3, 50 mmol/l KCl, 1.5–4.5 mmol/l $MgCl_2$, 50 mmol/l dNTPs, a 0.25-mmol/l concentration of each primer, 100 ng of genomic DNA and 1 U of BioThermStar™ DNA polymerase (Gene Craft GmbH, Münster, Germany). The PCR cycling reaction was initiated with a 10-min denaturation step at 95 °C to activate BioThermStar DNA polymerase. Subsequent denaturing steps included 94 °C for 20 s and an extension step of 72 °C for 45 s. For analysis of possible mutations within the promoter region and all exons including splicing sites of the APC gene, primers were designed to amplify the promoter region and each exon including the splicing acceptor and donor sequences. Primer pairs and the adjusted temperatures for individual amplicons are given in tables 1 and 2.

Denaturing High-Performance Liquid Chromatography

Denaturing high-performance liquid chromatography (DHPLC) was carried out on automated HPLC instrumentation equipped with a DNASep column (Transgenomic Inc., San Jose, Calif., USA). The column was packed with proprietary 2-mm nonporous alkylated poly(styrenedivinylbenzene) beads [16]. The mobile phase was 0.1 M triethylammonium acetate buffer, pH 7, containing 0.1 M EDTA. Crude PCR products were subjected to an additional denaturing step at 95 °C for 3 min followed by a gradual reannealing step from 95 to 65 °C over a period of 30 min. The products were eluted with a linear acetonitrile (9017-03; J.T. Baker, Phillipsburg, N.J., USA) gradient at a flow rate of 0.9 ml/min. The start and end points of the gradient were adjusted according to the size of the PCR products using an algorithm provided by WAVemaker system control software (Transgenomic Inc.). Eight microliters of PCR product was injected for analysis in each run. Generally, analysis took about 10 min per injection, including column regeneration and reequilibration to the starting condition. The temperature required for successful resolution of heteroduplex molecules was determined with the DHPLC melting algorithm. Details about the temperature conditions and the algorithm itself were obtained using WAVemaker software. The appropriate experimental conditions for performing DHPLC are listed in tables 2 and 3. Heterozygous profiles were identified by visual inspection of the chromatograms on the basis of the appearance of additional earlier eluting peaks. Corresponding homozygous profiles showed only 1 peak.

Table 1. Primers and conditions for DHPLC (promoters and exons 1–14)

Amplicon	Forward primer (5'-3')	Reverse primer (5'-3')	Size bp	Annealing, °C	DHPLC °C	Gradient ¹
PROMOTER-A	CCCCCATTTAAACCAGAAATTT	TCGATTGCTATGATTTTTTGACG	541	58	54	59% B 4.5 min
PROMOTER-B	TCATCACTCTGACAACCTCAGTGAC	ACCTACCCCATTTCCGAGTC	509	58	60	59% B 4.5 min
APCe1	TTTCTTTAAAAACAAGCAGCCA	CACAGAAAACCTTGCCCTCAG	519	58	53	60% B 4.5 min
APCe2	AAGGTGCGTGCTTTGAGAGT	ACCAACACCCAAATCGAGAG	308	58	54	57% B 4.5 min
APCe3	CCAAGTGGACTTTTCAGGGA	CTGGAGTACACAAGGCAATGTT	412	58	53	57% B 4.5 min
APCe4	GCTCTTCTGCAGTCTTTATTAGCA	CCTAGTTGAACCTTGAGGTCC	444	58	53	58% B 4.5 min
APCe5	AAGCCACTTGTTTCCGGTTC	GTGTCTCAGCAGCCATGATA	511	58	54	58% B 4.5 min
APCe6	TGCGGTGAGCTGAGATTATG	ACCCACAAACAAGAAAGGCA	376	58	54	57% B 4.5 min
APCe7	GCAGCTCTAATGCTCAAGGG	TGGTACTGAATGCTTCTGGAAA	465	58	57	54% B 4.5 min
APCe8	CCATTCTGCAGTTTAATGCTCA	TAGAGATGGGGTTTTGCCAC	512	58	53	60% B 4.5 min
APCe9	CTGGAAAAGTTTCCGGTTC	TGCTTTGAAACATGCACTACG	577	58	58	57% B 4.5 min
APCe10	GTCAAGGGCAGATGAGTGGT	TTCTATGCTGGAACCAGGG	511	58	54	59% B 4.5 min
APCe11	TTGTCTTTTCTCTCTGCCC	AGCGAATGTGAAGCACAGGT	362	58	54	57% B 4.5 min
APCe12	CCTGTTGCTTATCATTTCTCACC	AGAGTGAGACCTGCCTCAA	391	62	54	57% B 4.5 min
APCe13	CAGCCTCCCAAAGTGATAGG	ATGGCTAAAAGAGGCAGCA	341	58	54	56% B 4.5 min
APCe14	AGGGACGGGCAATAGGATAG	CATTGCTTACAATTAGTCTTTTTGA	404	58	54	57% B 4.5 min

¹ Eluent B: 0.1 M triethylammonium acetate, pH 7.25, % acetonitrile.

Table 2. Primers and conditions for DHPLC (exon 15)

Amplicon	Forward primer (5'-3')	Reverse primer (5'-3')	Size bp	Annealing, °C	DHPLC °C	Gradient ¹
APCe15-1A	AGAGTGGCACCCAACCATAG	TCCCATATGCTTCCTGGTC	526	58	54	60% B 4.5 min
APCe15-1B	CAGGCAAATCCTAAGAGAGAACA	CTTGATGAAGAGGAGCTGGG	558	58	56	60% B 4.5 min
APCe15-1C	GCTCAAGCTTGCCATCTCTT	TATGGGCAGCAGAGCTTCTT	552	58	56	61% B 4.5 min
APCe15-2A	CCAGGAACCTTCTCAAAGCG	GTGAAGGACTTTGCCTTCCA	521	58	55	60% B 4.5 min
APCe15-2B	GTCAATACCCAGCCGACCTA	AGGCTGATCCACATGACGTT	536	58	55	60% B 4.5 min
APCe15-2C	TTCCAACCACATTTTGGACA	GAGCTGATTCTGCCTCTTGG	429	58	56	59% B 4.5 min
APCe15-3A	AACGTCATGTGGATCAGCCT	TGCTGGATTGTTTCTAGGG	498	58	56	60% B 4.5 min
APCe15-3B	CAGACGACACAGGAAGCAGA	GCAGCTTGCTTAGGTCCACT	542	58	59	60% B 4.5 min
APCe15-3C	GTGAACCATGCACTGGAATG	TGTTGGCATGGCAGAAATAA	536	58	58	57% B 4.5 min
APCe15-4A	TTTGCCACGGAAAGTACTCC	TATCATCCCCCGGTGTAAAA	442	58	56	59% B 4.5 min
APCe15-4B	CTGTGGCAAGGAAACCAAGT	TGATTTTTGTGGGTGCAGA	481	58	58	59% B 4.5 min
APCe15-4C	CCCAAAGGGAAAAGTCACAA	GCTGATTGTTGGTTGGAGGT	524	58	55	60% B 4.5 min
APCe15-5A	TCACCTCATATTACACGCC	GGTTCTCCCTGTGAGTCAGG	473	58	56	59% B 4.5 min
APCe15-5B	ACTCCGGTTTGCTTTTCTCA	AGCAGCAGCAGCTTGATGTA	489	58	56	59% B 4.5 min
APCe15-5C	GCCTTCAAGACTCAAGGGTG	TTGTCCTGCCTCGAGAGATT	533	58	55	60% B 4.5 min
APCe15-6A	GCTGCTGCTGCATGTTATC	TGGCAACAAGGGCTTAATTCT	505	58	56	60% B 4.5 min
APCe15-6B	AATCTCTCGAGGCAGGACAA	TCCTTTGGAGGCAGACTCAC	532	58	57	60% B 4.5 min
APCe15-6C	CAGGTTTATCCAAGAATGCCA	TCAGAATGAGACCGTGCAA	458	58	56	59% B 4.5 min
APCe15-7A	CCCACCTAATCTCAGTCCCA	CAATCACCGGGGAGTATTA	530	58	56	60% B 4.5 min
APCe15-7B	AAATGGCACCTGCTGTTTCT	TTCCACTGGATTCTGTGCTG	546	58	57	60% B 4.5 min
APCe15-7C	TTGGAAAATCGCCTGAAGTC	TGGCTTCCAGAACAAAAACC	524	58	53	60% B 4.5 min

¹ Eluent B: 0.1 M triethylammonium acetate, pH 7.25, % acetonitrile.

Direct Sequencing

Sequencing reactions were performed for the samples that showed heterozygous profiles during DHPLC analysis using the ABI PRISM™ Dye Terminator Cycle Sequencing Ready Reaction kit (PE Applied Biosystems Inc., Foster City, Calif., USA). Electrophoresis was carried out using a Genetic Analyzer 310 (PE Applied Biosystems Inc.) equipped with long-read sequencing capillary and POP-6 sequencing polymer (PE Applied Biosystems Inc.). Primers used for direct sequencing reactions were the same as in PCR reactions (tables 1, 2).

Results

Of the 6 probands (2 male, 4 female) subjected to molecular analysis of the APC gene, mutations were detected in 3 (50%). Age at the diagnosis of disease ranged from 27 to 54 years, with a mean of 39 years. All 6 of these probands had more than 100 adenomas in the colon and

Table 3. Summary of the APC gene sequence changes in this study

Designation	Heterozygous frequency ¹		Allelic frequency in FAP probands ¹
	FAP probands	control population	
c.4660-4661insA	1/6	0/250	(A) ₆ /(A) ₆ : 5/6; (A) ₆ /(A) ₇ : 1/6; (A) ₇ /(A) ₇ : 0/6
c.5911C>G	1/6	0/250	C/C: 5/6; C/G: 1/6; G/G: 0/6
c.6495C>T	1/6	0/250	C/C: 5/6; C/T: 1/6; T/T: 0/6
c.136-53T>C	2/6	47/250	T/T: 4/6; T/C: 2/6; C/C: 0/6
c.646-140G>T	1/6	31/250	G/G: 5/6; G/T: 1/6; T/T: 0/6
c.1959+7T>C	3/6	26/250	T/T: 2/6; T/C: 3/6; C/C: 1/6
c.1955-142-143insAGAA ²	3/6	68/250	(AGAA) ₂ /(AGAA) ₂ : 3/6; (AGAA) ₂ /(AGAA) ₃ : 3/6; (AGAA) ₃ /(AGAA) ₃ : 0/6
c.2214G>A	1/6	78/250	G/G: 5/6; G/A: 1/6; A/A: 0/6
c.5879G>A	1/6	73/250	G/G: 5/6; G/A: 1/6; A/A: 0/6

¹ As DHPLC detects the presence of heteroduplex DNA, the heterozygous frequency instead of the specific allelic frequency is shown in this table. The allelic frequency in the FAP probands is listed according to the sequencing results.

² This polymorphic site indicates the changes in the repeat number of the AGAA tandem sequence.

had received total colectomy. Two of them had invasive lesions (rectal cancer in one, transverse colon cancer in the other). As for the extracolonic manifestations, 1 had fundic gland polyposis and another had lipoma on the back. In addition, a third proband had a family member who suffered from oral cancer.

In this study, we used DHPLC as the first screen to identify heterozygous profiles and found 21 heterozygous profiles. Among the 21 heterozygous profiles, 3 had amino acid changes which occurred only in the FAP probands and not in the 250 population controls. These three mutations were all in exon 15. Two of these mutations were novel, while the third belonged to a category that has been reported as a mutation hot spot in APC. The first novel mutation was at codon 2166 in exon 15, with a C to T transition (CGA → TGA; c.6495C>T), resulting in a stop codon (fig. 1a). The second novel mutation we found was at codon 1971 in exon 15, with a C to G transversion (TCT → TGT; c.5911C>G), resulting in an amino acid change from serine to cysteine (fig. 1b). The third mutation involved an A insertion in an -AAAAA- sequence at codons 1554–1556 (c.4660-4661insA) (fig. 1c), which caused a stop codon downstream (at codon 1558). Another 2 heterozygous profiles had nucleotide changes in the exons (c.2214G>A, c.5879G>A) that were also detected in the control population and were hypothesized to represent polymorphism. Another 9 heterozygous profiles had 4 types of base substitution in the intron sequences that did not affect the splicing sites. No sequence change was identified in the remaining 7 heterozygous profiles detected by DHPLC. The locations and frequencies of these

sequence changes are summarized in table 3. The nomenclature for the description of sequence variations was guided by a new nomenclature system recommended by the Human Genome Variation Society (<http://www.hgvs.org/mutnomen/>) for a uniform and unequivocal description of sequence variants.

Discussion

This study is the first report of mutation analysis of the APC gene in Taiwanese. Among the 6 probands subjected to genetic analysis of the APC gene, 3 mutations (50%) were detected. Compared with previous studies [11, 17, 19, 20, 24, 25], the mutation rate in this study is similar to those reported for Israelis (50.7%) and Czechs (45.4%), but lower than those for Greeks (83%), Finns (72%) and others (up to 95%). The mutation rates in the APC gene vary among different ethnic groups. Further comparison of Chinese ethnic groups from different areas showed that our result in Taiwanese was similar to that from Hong Kong (50%), but lower than that from Singapore (68%) [5, 26]. In contrast to the Hong Kong report and our study, which included exclusively ethnic Chinese, the study from Singapore included some patients from different ethnic groups. This might explain the discrepancy between the APC mutation rates in Chinese ethnic groups from different areas.

After searching the APC mutation databases (UMD) [31], we found that two of the three mutations that we identified were novel. These two mutations were located at codons 1971 and 2166, which were predicted to be the

Fig. 1. The APC mutations detected in FAP probands. The upper panels show heterozygous profiles detected by DHPLC. The automatic sequencing results are shown in the lower panels. **a** The C to T transversion (CGA → TGA) resulted in a stop codon at codon 2166 in

exon 15 (c.6495C>T). **b** The C to G transition (TCT → TGT) resulted in an amino acid changing from serine to cysteine at codon 1971 in exon 15 (c.5911C>G). **c** An A insertion at codons 1554–1556 (c.4660–4661insA) caused a stop codon downstream (codon 1558).

binding sites for β -catenin and axin, respectively [9]. The third mutation was located in an area that was previously reported as a mutation hot spot for APC (codon 1556) [10, 22, 23, 33] and was also predicted to be the binding site for β -catenin [9]. Mutations in these areas could potentially cause a disruption of interactions between APC and β -catenin or axin, which in turn would block APC's role as a gatekeeper, hence causing cancer. Whether these mutations will cause incompetence of protein-protein interaction between APC and β -catenin or axin remains to be tested. All three mutations were located in exon 15, which was in accordance with previous reports that exon 15 comprises three fourths of the coding sequence of APC and is the most common target for both germ line and somatic mutations [9]. Notably, mutation in the most common germ line hot spots (codons 1061 and 1309) [9] was not found in our series.

Since only 50% of FAP probands have a mutation in the APC gene detected by DHPLC followed by direct nucleotide sequencing methods, we performed another

two studies to further clarify the genetic background of Taiwanese FAP probands. First, a protein truncation test was performed to check whether a large fragment deletion existed, such as whole exon deletion, which sometimes escapes DHPLC detection. According to the protein truncation test results, no large deletion or truncated protein was detected (data not shown). Hence, DHPLC seemed to be a sensitive and efficient test for mutation analysis in this study, as has been previously noted [34, 35]. The second examination we performed was to check the NH₂-terminal regulatory domain of the β -catenin gene in probands lacking APC mutations. Somatic mutations in the NH₂-terminal regulatory domain of β -catenin were found in 48% of colorectal cancers lacking APC mutations [32]. PCR using genomic DNA and primers as described previously [32] followed by direct sequencing with internal primers did not find any nucleotide change in the 3 probands. A similar condition had been reported previously [4, 7]. The genetic cause of the disease in these 3 probands remains to be elucidated.

Genetic analysis is the basis for early diagnosis and chemoprevention of FAP. For the first time in Taiwan, we were able to identify mutations in Taiwanese FAP patient kindreds and to perform molecular diagnosis. Future studies should enroll more FAP probands to establish the genetic basis of FAP in Taiwanese. To confirm that the mutations are germ line mutations, other members of the FAP probands' families should be included. However, some of the family members were not available at the time of this study. Analysis of other family members is needed. Germ line MYH mutation should also be exam-

ined when the family history is compatible with recessive inheritance [30]. If the mutation rate of the APC gene is still low, linkage analysis of the negative mutation screening kindreds should be considered.

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