A nonsense mutation in exon 8 of the *APC* gene (Arg283Ter) causes clinically variable FAP in a Malaysian Chinese family

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(Received April 7, 2003/Revised June 16, 2003/Accepted June 18, 2003)

The present study was carried out to characterize the causative genetic mutation in a medium-sized Malaysian Chinese pedigree of three generations affected with familial adenomatous polyposis (FAP). Clinical data and genetic studies revealed considerable phenotypic variability in affected individuals in this family. Blood was obtained from members of the FAP-01 family and genomic DNA was extracted. Mutation screening of the *adenomatous polyposis coli* (*APC*) gene was carried out using the single strand conformation polymorphism (SSCP) technique. The possibility of exon skipping was predicted by splicing motif recognition software (ESEfinder release2.0). SSCP results showed mobility shifts in exon 8 of the *APC* gene which segregated with affected members of the family. Sequence analysis revealed that the affected individuals are heterozygous for a C847T transition, whilst all the unaffected family members and control individuals are homozygous C at the same position. This nucleotide substitution generates a stop codon at amino acid position 283, in place of the usual arginine (Arg283Ter). We conclude that an Arg283Ter mutation in the *APC* gene is causative of the FAP phenotype in this family, although there is considerable variation in the presentation of this disease among affected individuals. Computational analysis predicts that this mutation occurs within sequences that may function as splicing signals, so that the sequence change may affect normal splicing. (Cancer Sci 2003; 94: 725–728)

Familial adenomatous polyposis (FAP) is an autosomal dominant condition that is characterised by the development of hundreds to thousands of adenomatous polyps in the colon, beginning during the second or third decade of life. Untreated, colorectal cancer invariably develops and death from carcinoma is expected at a mean age of 42.[1)] Reported incidences of FAP range from 1 in 7000 to 1 in 16,000 worldwide.[2)] In Malaysia, surveys have indicated that colorectal carcinoma is the second most common cause of cancer death in both males and females.

FAP is caused by defects in the *adenomatous polyposis coli* (*APC*) gene. The first reports appeared in 1991,[3,4)] and since then numerous mutations have been identified in the *APC* gene. Exon 15 comprises >75% of the coding sequence of the *APC* gene and is the most common target for both germline and somatic mutations. APC mutations are generally accepted as highly penetrant (close to 100%[5)]), although there is striking variation in phenotypic expression of the disease.[6,7)] Clinically, it has been suggested that the absence of clinical features by the age of 40 years would indicate non-carrier status of the APC mutation.[8)]

Materials and Methods

Clinical data. Fig. 1 shows the pedigree of a Malaysian Chinese family (FAP-01) diagnosed with FAP. The propositus (II.2) was 43 years of age when she was diagnosed with colorectal cancer with multiple colonic adenomatous polyps. The diagnosis of FAP was made when her sister (II.14) was also diagnosed with colorectal cancer with multiple polyps at the age of 33 years. This patient underwent colectomy but succumbed to her illness when she developed secondaries to the liver. Further communication with family members revealed that their father had a history of irritable bowel symptoms (I.1) and had died at the age of 43 years from unknown causes. Other family members were later advised to opt for pre-symptomatic screening by colonoscopy. Siblings II.10 and II.11 were asymptomatic, at the ages 37 and 38 years, respectively, but colonoscopy revealed the presence of multiple polyps. These individuals were advised to undergo preventive colonic surgery. Sibling II.5 was asymptomatic with no polyps seen on first endoscopy at the age of 44 years. However follow-up endoscopy 3 years later revealed the presence of a few polyps. He is currently 53 years old and well, and has not undergone surgery. Sibling II.4 is well and no polyps were seen endoscopically. Sibling II.7 is asymptomatic and had declined endoscopic examination. All of the propositus' children (III.1, III.2 and III.3) had colonoscopies done at ages 26, 20 and 16 years, respectively, and were found to have multiple adenomas. Patient III.16 was also found to have multiple adenomas. All of these patients had elective colectomy soon after diagnosis.

It is noteworthy that sibling II.10 was also diagnosed with papillary carcinoma of the thyroid and was advised to undergo thyroidectomy. This condition was also seen in her daughter (III.16) and her niece (III.3). All of them underwent thyroidectomy.

Genetic screening. Mutation screening of the *APC* gene was initially performed using the single strand conformation polymorphism (SSCP) analysis technique and direct sequencing. After identification of the *APC* gene mutation, a PCR-RFLP based assay was designed to test all family members for whom DNA samples were available. Screening of unaffected control samples representing the general population was also carried out to assess the presence of the APC mutation in the population.

Collection of samples and DNA extraction: DNA samples were obtained from 30 members of family FAP-01. DNA extraction was carried out using established methods. The complete coding region of the *APC* gene was amplified in a total of 31 fragments using primer information described by Groden *et al*.[3)] PCR optimization and amplification were done using standard techniques and equipment.

SSCP analysis: SSCP analysis was performed using "MDE" gels. Electrophoresis was carried out using the Protean II electrophoresis cell system with a temperature-regulated water circulator for temperature control. For each PCR fragment, SSCP

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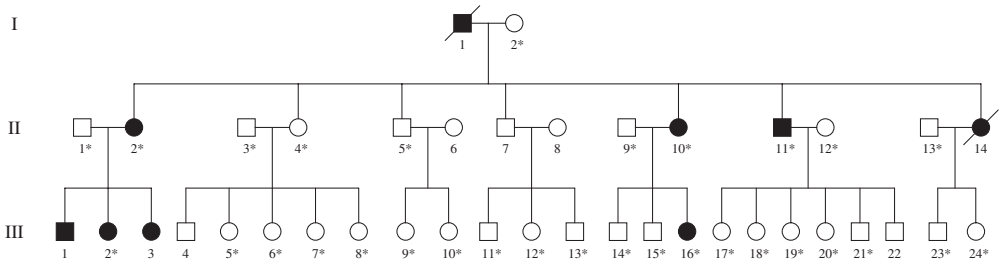

Fig. 1. Family FAP-01. Solid boxes and circles represent affected males and females respectively. Asterisks indicate individuals included in this study.

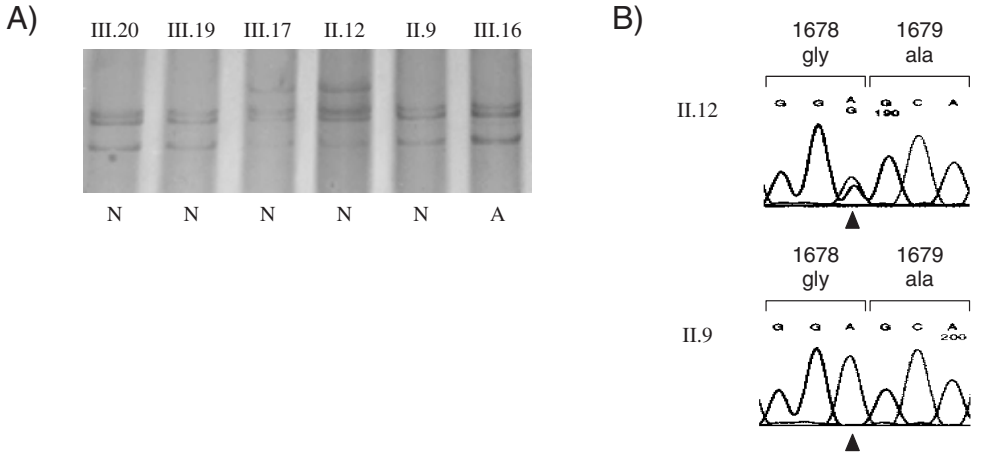

Fig. 2. SSCP variants and sequence analysis of exon 15J. A) SSCP banding variation was not consistent with disease status, and therefore was concluded to be non-pathogenic. N, unaffected; A, affected. B) Sequencing of variant bands revealed a heterozygous A to G transition at the third base of codon 1678 which does not alter the amino acid sequence.

optimization was carried out by repeating the electrophoresis using at least 2 different gel concentrations (0.5× and 0.75×) and at least 2 different electrophoretic temperatures (10°C and 20°C). This process is also advantageous as it increases the sensitivity of the SSCP techniques to detect mutations.

Sequencing analysis: Following SSCP screening, PCR fragments showing mobility shifts (variations in SSCP banding pattern) were selected for sequencing in order to determine the nature of the sequence change. Purification of PCR products was performed using commercially available purification kits. The purified products were subjected to cycle sequencing reactions using the Big Dye Terminator Cycle Sequencing version 2 kit (PE Applied Biosystems, Foster City, CA), and electrophoresis was done on an ABI310 Genetic Analyser.

NlaIII PCR-RFLP assay: The sequence change identified creates a new *Nla*III (CAT↓G) restriction site, allowing the C and the T allele to be unequivocally assigned by a simple assay. The fragment containing the mutated region was amplified by PCR followed by enzyme digestion, carried out as recommended by the manufacturer. For the C allele, the 185-bp PCR product will be cleaved into two fragments of 115 bp and 70 bp, respectively. For the T allele, the 70-bp fragment will be further cleaved into two fragments of 59 bp and 11 bp, respectively. For better resolution the digested product was electrophoresed in a 15% polyacrylamide gel followed by ethidium bromide staining to visualize the digested products.

Prediction of splicing motif recognition sequence: Prediction of the presence of a splicing motif sequence was done using web-based ESE prediction software.[9)]

Results

SSCP results for exon 15J showed shifts in banding pattern in different individuals. However, the variation was not patient-specific, and subsequent sequence analysis revealed a silent mutation at the third base of codon 1678 (A to G) that does not alter the amino acid glycine (Fig. 2).

SSCP analysis of exon 8 showed SSCP mobility shifts that were consistent with the pattern of disease segregation (Fig. 3). Subsequent sequence analysis of exon 8 revealed a C to T transition in the first base of codon 283 in one of the alleles, which is predicted to introduce a premature stop codon at that position (Arg283Ter). This mutation was detected in affected individuals in the second generation and not in their spouses. Because this mutation creates a new *Nla*III site, we were able to design a simple PCR-RFLP assay to screen for the presence of this mutation in all available family members, as well as in a panel of 100 control individuals. Using this assay, we confirmed that all of the affected members of this family carry the nonsense mutation in a heterozygous state, and that this mutation is not present in at least 100 control individuals that were tested. We were also able to identify additional family members (III.14, III.23 and III.24) who have inherited the mutant allele, but who are as yet undiagnosed (possibly due to their young age). The only discrepancy seen in the predictive test results is with individual II.5, who has been classified as unaffected, but carries the nonsense mutation. A repeat sample obtained from that individual showed similar results.

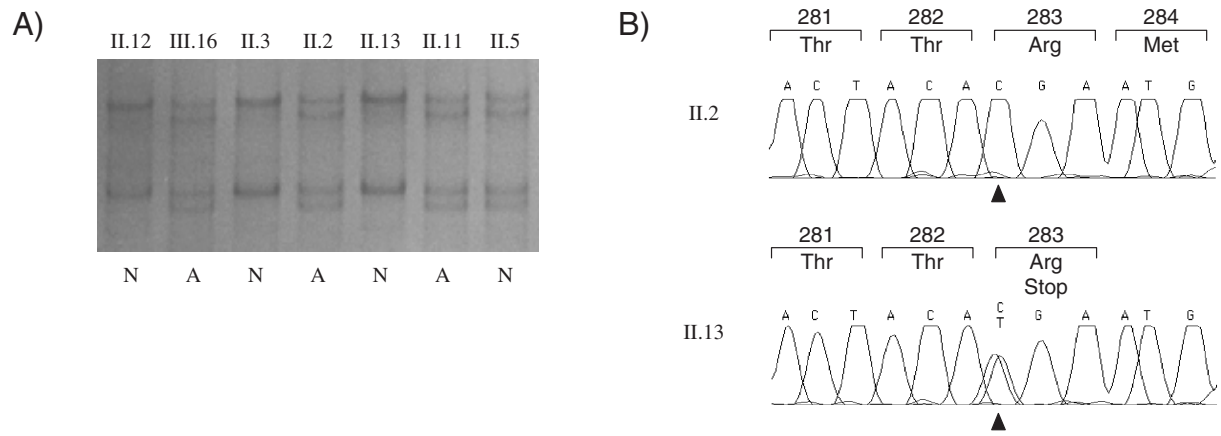

Fig. 3. SSCP variants and sequence analysis of exon 8. SSCP banding variation was consistent with disease status, except for individual II.5 (see "Discussion"). N, unaffected; A, affected. Sequencing of variant bands revealed a heterozygous C to T transition at the first base of codon 283 which results in a termination codon.

Table 1. Predictive score matrices obtained using the ESE prediction software

	Motif : TACACGA SR protein : SF2/ASF		Motif : TACACG SR protein : SRp55
A)	Score	B)	Score
TACACGA (normal allele)	3.6386	TACACG (normal allele)	0.0000
TACATGA (mutant allele)	0.0000	TACATG (mutant allele)	3.1552

A) The motif TACACGA is a putative binding site for SF2/ASF protein, which is abolished in the mutant allele. B) Conversely, a new SRp55 binding site is predicted in the mutant allele.

Discussion

In this study we identified an Arg283Ter mutation located in exon 8 of the *APC* gene, causing FAP in a three-generation Malaysian Chinese kindred. A similar mutation was reported by Pang *et al.*[10] for another Chinese family from Hong Kong segregating FAP associated with a variant of congenital hypertrophy of the retinal pigment epithelium (CHRPE). Clinical evaluation of our patients however did not indicate the presence of CHRPE in any of the patients, which is consistent with observations by Olschwang *et al.*,[11] who noted that CHRPE lesions were almost always absent if the mutation occurred before exon 9. On the other hand, out of the five patients diagnosed with FAP, three of the affected family members also presented with papillary cancer of the thyroid, and had been advised to undergo thyroidectomy. FAP-associated thyroid carcinoma has indeed been reported in the literature as one of the extracolonic manifestations of FAP, although the exact incidence has not yet been established.[12, 13] The authors suggested that *APC* gene mutations may increase susceptibility to thyroid carcinoma and that other genes or environmental factors are required for its presentation. It is also interesting to note that one family member (II.5), aged 53, was reported to be asymptomatic. Such instances have been reported[14, 15] and represent one of the main challenges in genotype-phenotype correlation in FAP studies. Other than influences from environmental factors, the presence of modifier genes[16] that may influence the severity of FAP has been suggested as a possible contributor to phenotypic variation.

The predicted outcome of the Arg283Ter mutation is a nonfunctional COOH-terminally truncated protein which is only 282 amino acids long and lacks almost 90% of the normal protein (the most common normal APC protein isoform is composed of 2843 amino acids). The presence of one wild-type allele however is thought to be sufficient to retain normal APC function in non-neoplastic cells, although the wild-type APC allele is often lost in most colorectal tumors of both sporadic and FAP patients.[17] Several studies have suggested that mutations in the 5-prime end of the *APC* gene are associated with an attenuated phenotype, although there is considerable intra- and interfamilial variation in the presentation of the phenotype seen among the affected patients.[18–20]

The common presumed outcome of nonsense mutations is truncated protein isoforms. Though this may be correct in most instances, it can be misleading in others, especially in cases where the mutation affect sequences that represent signals for other cellular processes. Nonsense mutations that result in exon skipping have been documented in Marfan syndrome (exclusion of exon 51 of the *fibrillin 1* gene),[21] Becker muscular dystrophy (exclusion of exon 27 of the *dystrophin* gene)[22] and predisposition to breast and ovarian cancer (exclusion of exon 18 of the *BRCA1* gene).[23] In all three cases, the nonsense (point) mutations disrupt an exonic splicing enhancer (ESE) sequence within the particular exon, preventing recognition and binding of SR proteins (a group of splicing factors); this would then result in the exclusion of the exons from the mature transcript. The fate of the translated product then depends on whether or not the reading frame has been retained in the variant transcripts following the aberrant splicing.

We attempted to predict if the Arg283Ter mutation would result in exon skipping by employing splicing motif recognition computer programmes. We used web-based ESE prediction software[9] that assigns score matrices to putative ESE sequences for different splicing factors (SR proteins) and two opposing predictions were obtained. On the one hand, it was predicted that the C to T transition mutation of the TACACGA motif would actually abolish an SF2/ASF binding site (as shown by the elimination of the SF2/ASF score matrix in Table 1). This would theoretically result in skipping of exon 8 from the mature transcript, and since the resulting reading frame would still be retained during translation, the end protein product would be expected to be an isoform deficient in only 23 amino acids compared to the original protein. On the other hand, the mutation is also predicted to create a 'new' SR protein binding site (SRp55). The outcome would then depend on whether SRp55 could function as effectively as SF2/ASF, thus allowing normal splicing to be retained.

Futher study should provide insights into the pathogenic mechanisms of the APC mutation in this family, and perhaps in other FAP cases.

This project is supported by the RMK7 IRPA Research Grant 06-02-03-0775 and University of Malaya F vote. We thank all the family members who have participated in this study. We appreciate the technical contributions of Ms. Salehah Saruman. We also thank Prof. Neva Haites, Medical Genetics, Foresterhill, Aberdeen, for providing the initial primer sets.

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