

A novel aberrant splice site mutation in the APC gene

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Familial adenomatous polyposis (FAP) is an inherited, autosomal dominant syndrome characterised by the presence of multiple (>100) adenomatous polyps in the colon and rectum. These polyps, if left untreated, progress to colorectal cancer (CRC), typically by the age of 40 years.¹ Other clinical features include variable age of onset of polyposis (age 10–40 years) and variable expression.² FAP accounts for about 1% of all colorectal cancers.^{3,4} In addition to colonic polyps, FAP patients may present premalignant lesions in the upper gastrointestinal tract, extraintestinal manifestations such as osteomas and epidermoid cysts, desmoid formation, congenital hypertrophy of the retinal pigment epithelium (CHRPE), and other malignant changes, such as small bowel cancer and tumours of the brain and thyroid gland.²

FAP is caused by the dominant inheritance of germline mutations of the adenomatous polyposis coli (APC, MIM 175100) tumour suppressor gene.^{3–6} APC is a 312 kDa protein translated from a major transcript consisting of 15 exons with the last exon occupying 77% of the total protein. Additional APC transcripts occur because of alternative splicing of exons 3–4, 9, 10A/X, and 14.^{7–9} The majority of APC mutations result in the formation of a truncated protein¹⁰ and most germline mutations are reported in the first half of the gene.^{11–13} Mutant APC proteins lack the potentially important functional motifs including binding domains for β -catenin, microtubulin, and EB1.^{14–16} Furthermore, the armadillo repeats are located between APC exons 10–14, and deletion of this domain disrupts interactions between APC and other partner proteins including PP2A and ASEF.^{17,18}

The GT and AG sequence motifs are highly conserved consensus splice donor and acceptor site sequences at the intron-exon boundaries.^{19,20} Splice site defects account for approximately 15% of disease causing point mutations of various genes.²¹ The majority of splice site mutations consist of splice donor (SD) site defects. The splice acceptor (SA) site mutations mostly involve the invariant AG sequence and typically cause exon skipping. Only about 13% of all SA mutations result in the creation of novel splice sites.

Truncating APC mutations mostly include nonsense point mutations or small insertions/deletions (<http://perso.curie.fr/Thierry.Soussi/APC.html>). To date, few SA mutations have been reported for APC. Published reports have either shown SA mutations resulting in exonic deletions²² or have not characterised SA substitutions further, simply citing them as “splice acceptor defect”.²³ Here we describe a family with a classical polyposis phenotype that harbours a germline SA mutation in the invariant AG sequence of APC exon 8, generating a novel cryptic splice acceptor site immediately downstream. This splicing defect results in a single base pair deletion at the beginning of the exon at the transcriptional level and leads to a premature truncation within APC exon 8.

PATIENTS AND METHODS

Selection of patients

This kindred was referred to the molecular diagnostic laboratory at the Department of Pathology and Laboratory Medicine, Mount Sinai Hospital, as part of a provincial predictive genetic

screening programme for FAP. Affected subjects had the classical polyposis phenotype, characterised by numerous adenomatous polyps. Endoscopic, operative, and histopathological data were collected retrospectively on all affected patients and complete follow up information was obtained as well. Predictive genetic testing was offered to clinically affected and at risk subjects, with pre- and post-test genetic counselling as described previously.²⁴ Patient accrual, sample collection, and genetic screening were performed according to guidelines of the Ethics Committee of the University of Toronto.

Molecular genetic analysis

The protein truncation test (PTT) assay was initially used to screen for underlying germline APC mutations in this kindred.²⁵ Blood samples were obtained from patients and lymphocytes were isolated using NH₄Cl-Tris (1.4 mol/l NH₄Cl, 17 mmol/l Tris, pH 7.6). Samples were mixed by inversion, incubated at 37°C for 25 minutes, and spun at 1600 rpm for 10 minutes. The WBC pellet on ice was resuspended in PBS and spun at 1600 rpm for 10 minutes. RNA extraction was performed using TRIzol according to the manufacturer's protocol (Invitrogen Life Technologies, Burlington, Canada), and DNA extraction was performed using a saturated salt solution.²⁶ Reverse transcription PCR was performed by use of standard techniques. In brief, cDNA was generated from total RNA (5 μ g) by use of random hexamers pd(N₆) (Amersham Pharmacia, Piscataway, NJ), oligodT (Invitrogen Life Technologies), 1 \times first strand buffer, 0.5 mmol/l of deoxynucleotide triphosphate, 5 mmol/l DTT, and 200 units of SuperScript II (Invitrogen Life Technologies). cDNA was synthesised by incubating at 45°C for one hour followed by 70°C for 15 minutes.

APC exon 15 was amplified in four overlapping segments, as described previously.²⁵ Exons 1–14 were amplified in two overlapping segments: 1A (exons 1–9) and 1B (exons 8–14). The 5' end of each forward primer had a T7 promoter sequence and a translation initiation site (5' GGA TCC TAA TAC GAC TCA CTA TAG GGA GAC CAC C 3') for coupled in vitro transcription and translation. Amplification of the region (segment 1A) that harboured the APC mutation in this kindred was performed using primers SIIF (5' ATG GCT GCA GCT TCA TAT GAT C 3', APC nucleotides 1–22) and 9AR (5' AGC CTC TTT ACT GCC CCG 3', APC nucleotides 1120–1137). PCR conditions were as follows: initial denaturation at 95°C for two minutes; 35–40 cycles, each consisting of denaturation at 95°C for 30 seconds, annealing at 63°C for one minute 30 seconds, and extension at 70°C for two minutes; and a final extension at 70°C for five minutes. Five μ l aliquots of PCR products were electrophoresed on a 1.5% agarose gel, in order to confirm amplification.

Abbreviations: FAP, familial adenomatous polyposis; CRC, colorectal cancer, CHRPE, congenital hypertrophy of the retinal pigment epithelium; APC, adenomatous polyposis coli; SD, splice donor; SA, splice acceptor; PTT, protein truncation test; AAPC, attenuated APC

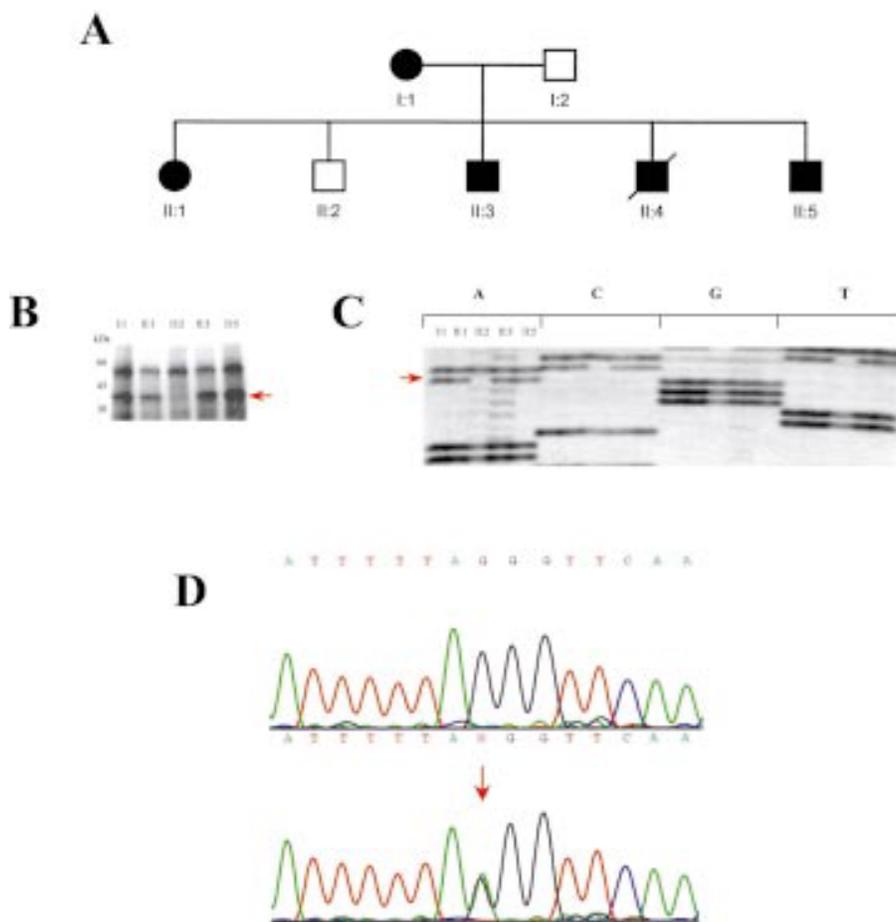


Figure 1 (A) Pedigree of the FAP family with a classical polyposis phenotype described in this study. Affected and unaffected members are shown as solid and open symbols, respectively. (B) PTT analysis shows APC protein truncation (33 kDa) for all affected members (patients I.1, II.1, II.3, II.5). (C) RT-PCR and sequence analysis with reverse primer (AP8R) shows a "G" deletion in all affected members. (D) Automated sequencing of genomic DNA shows G>A substitution in an affected member (bottom panel) compared to the wild type sequence (top panel).

The *in vitro* synthesised PTT assay was performed by use of a commercial kit (TNT T7 Quick Coupled Transcription/Translation System; Promega, Madison, WI), according to the manufacturer's protocol. In brief, TNT Quick Master Mix (Promega) and ^{35}S -methionine (Amersham Pharmacia) were added to the RT-PCR product and incubated at 30°C for 90 minutes. SDS loading buffer/B-mercaptoethanol (19:1) was added to the sample before a flash spin at maximum speed (14 000 rpm for two minutes). Protein products were electrophoresed on a 4-15% Linear Gradient Tris-HCl gel (Bio-Rad Laboratories, Hercules, CA). Positive PTT results were confirmed by two independent PCR reactions.

For sequence determination, RT-PCR products of the putative PTT positive samples were electrophoresed on a 1.5% agarose gel for 45-60 minutes at 100 V and were stained with ethidium bromide. The band of interest was excised with a sterile scalpel blade, and DNA was extracted by use of the QIAquick Gel Extraction Kit (QIAGEN Inc, Mississauga, Canada). The dideoxy mediated chain termination method was used for DNA sequencing.²⁷ For this purpose, we used α [^{32}P] dideoxynucleotide and a ThermoSequenase radiolabelled terminator sequencing kit (Amersham Pharmacia) with primers AP7F (5' CAT GGC AAC TTC TGG TAA TG 3', APC nucleotides 810-829), AP8R (5' CAC TGG CTG TTT CAT GGT C 3', APC nucleotides 853-871), and 9AR. Sequencing was performed according to the manufacturer's protocol. Finally, the gel was autoradiographed by use of BioMax MR film (Eastman Kodak Company, Toronto, Canada) for 24-48 hours at room temperature.

In order to confirm the mutation identified at the transcriptional level, genomic DNA sequencing was performed using intronic primers 8FN (5' TAA ATT ATA CCA TCT ATA ATG TGC 3', APC EX8-12 to EX8-35) and 8RN (5' GCA TTA GTG ACC AGG GTT TG 3', APC EX8+15 to EX8+34), flanking APC exon 8. Dideoxy sequencing of the PCR products was performed using a BIGDYE dideoxy sequencing ready reaction kit and analysed on an ABI 310 automated sequencer (PE Foster City, California, USA).

RESULTS AND DISCUSSION

This is the first report of a complex germline APC mutation caused by recruitment of a novel cryptic SA site resulting in familial polyposis. In this family, PTT analysis initially showed a truncated mutant APC protein in the germline of affected FAP patients (fig 1). RT-PCR and sequence analysis identified a "G" deletion at the beginning of APC exon 8 (nucleotide 834), resulting in a stop (TGA) in the same exon (codon 293). Further sequencing of genomic DNA, however, did not show the same "G" deletion as expected, but showed a G>A substitution mutation at -1 SA site of APC exon 8. This substitution results in the use of a cryptic splice acceptor site immediately adjacent to the mutation, which creates an apparent "G" deletion at the transcriptional level (fig 2). This germline mutation was identified in all clinically affected members of the family.

SA substitution mutations are usually predicted to result in an exonic deletion and therefore further investigations of the mRNA transcripts are not always carried out.²³ Genotype-phenotype correlative studies in FAP have shown that

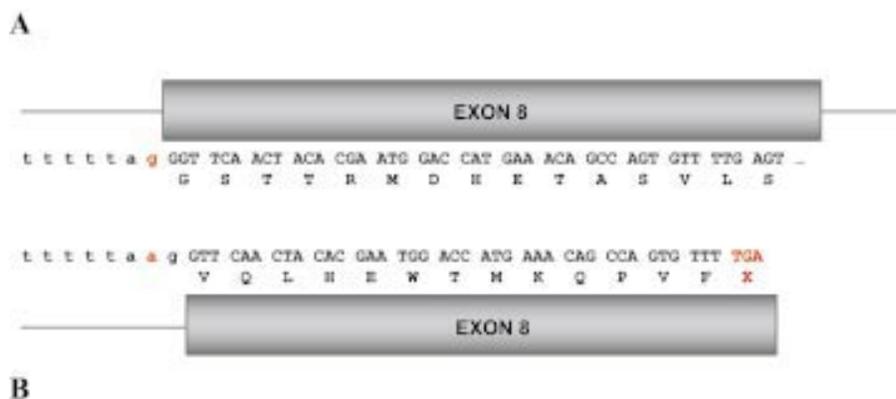


Figure 2 Illustration representing a cryptic splice site generated from a point mutation in the invariant “G” at the –1 position of the authentic splice acceptor site sequence of *APC* exon 8 (A). The cryptic splice site causes a single base frameshift resulting in altered amino acid sequence and a stop codon within the same exon (B).

germline mutations at the extreme 3' end of the *APC* gene that retain different functional domains lead to a milder form of FAP, also known as attenuated adenomatous polyposis coli (AAPC).²⁵ If SA substitution in this family were to cause exon skipping, then it would have resulted in an in frame deletion of *APC* exon 8. Such a mutant *APC* transcript would have maintained all the functional domains present in the wild type *APC* and would be predicted to result in AAPC. All affected patients from this family have presented with the classical polyposis phenotype, supporting the use of newly created cryptic splice acceptor site resulting in *APC* truncation within exon 8. Indeed, truncating mutations in *APC* exon 8 have been associated with the classical FAP phenotype.²⁸

A survey of mutation databases for other cancer predisposing genes (*BRCA1*, *BRCA2*, *p53*, *MLH1*, and *MSH2*)* as well as *APC*, indicates that splice site mutations account for ~5-12% of all mutations reported (excluding unclassified variants). Among these, very few splice acceptor (–1) G>A alterations have been reported. To our knowledge, the use of a cryptic splice site similar to the one observed in this family has never been reported for any of these genes.

It is challenging to derive an explanation for the preference of cryptic SA site over exon skipping in this family. Splice acceptor sites are much less conserved than splice donor sites. An invariant AG sequence, a pyrimidine rich track of 10-20 nucleotides upstream of this sequence,²⁹ and a conserved sequence element known as “the branchpoint”, 18-40 bp upstream of the 3' splice site,³⁰ are among the recognition elements required by the spliceosome assembly for efficient splicing of exons. Using these three recognition elements, Shapiro and Senapathy³¹ devised a method of ranking and scoring potential splice sites by comparing the observed splice site sequences of many genes to the consensus splice junction sequences. They suggested that a higher score correlated with a greater probability for the usage of the splice site under investigation. When we calculated the score for the *APC* IVS7-IG>A alteration, as expected, the score for the mutant SA site was lower than the score for the wild type splice acceptor site (67 versus 83). However, the score for the proposed cryptic splice site immediately downstream was higher than the mutant splice site (81 versus 67). Interestingly, despite a higher score of 90, the exon 9 SA site was not used to generate mutant *APC* transcript in this family. One possible reason for this apparent discrepancy may be the greater distance that spliceosome assembly would have to cover, relative to the cryptic splice acceptor site.

In summary, we identified a germline *APC* mutation in the conserved splice acceptor sequence that has activated a cryptic splice site over the often preferred deletion of the exon. Whether a similar mechanism underlies mutations in other genes remains to be investigated.

Electronic database information. *Mutation databases: *APC*: <http://perso.curie.fr/Thierry.Soussi/APC.html>. *BRCA1* and *BRCA2*: http://www.nhgri.nih.gov/Intramural_research/Lab_transfer/Bic/. *MLH1* and *MSH2*: <http://www.nfdht.nl/>. *P53*: <http://www.iarc.fr/p53/index.html>.

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