Short Report

APC promoter 1B deletion in familial polyposis – implications for mutation-negative families


Over 1500 adenomatous polyposis coli (APC) gene mutations have already been identified as causative of familial adenomatous polyposis (FAP). However, routine genetic testing fails to detect mutations in about 10% of classic FAP cases. Recently, it has been shown that a proportion of mutation-negative FAP cases bear molecular changes in deep intronic and regulatory sequences. In this study, we used direct sequencing, followed by multiplex ligation-dependent probe amplification (MLPA) of genomic DNA from family members, affected by classic FAP. We first reported the family as mutation negative. With the launch of a new version of MLPA kit, we retested the family and a novel full deletion of promoter 1B was detected. The exact breakpoints of the deletion were determined by array comparative genomic hybridization (CGH) and long range polymerase chain reaction (PCR), followed by direct sequencing. The total APC expression levels were investigated by quantitative polymerase chain reaction (qPCR) assay and allelic expression (ASE) analysis. The APC gene expression was highly reduced, which indicates causative relationship. We suggest that there is a significant possibility that APC promoter 1B mutations could be found in mutation-negative FAP patients. In the light of our findings it seems reasonable to consider targeted genetic re-analysis of APC promoter 1B region in a larger cohort of unsolved cases.

Conflict of interest

Herewith we declare that we have no conflict of interest.

Familial adenomatous polyposis (FAP) is an inherited condition leading to multiple intestinal polyps and predisposition to colon cancer. Most cases are caused by mutations in the adenomatous polyposis coli (APC) gene (MIM#611731), with penetrance close to 100% at the age of 40 (1, 2). Over 1500 mutations have already been identified, with some genotype–phenotype correlations published in the literature (3, 4). Two promoters (1A and 1B) of the APC gene initiate transcription from distinct sites, and multiple transcripts are generated by alternative splicing (5). Promoter 1B is the most distal promoter of the APC gene and is 586 bp long (Genbank accession: D13981). Promoter 1A is located approximately 30 kb downstream and is 865 bp long (Genbank accession: U02509). The major APC transcript has an 8538 bp open reading frame and is initiated by promoter 1A. Promoter 1B produces three transcripts, 1B1, 1B2 and 1B3.

Hypermethylation of promoter 1A, but not 1B has been readily demonstrated in various human tumours and FAP (6), suggesting that promoter 1B has a minor role in APC inactivation. Recently, mutations in
the non-coding parts of the gene including promoter regions and deep intron sequences have been reported as responsible for FAP. Deep intronic alterations were detected in up to 8% of the mutation-negative patients (7). Full promoter 1A deletion was found in the Old Colony Mennonites (8). Partial 320 bp promoter 1B deletion was described in a Swedish FAP family (9).

Below we describe a full promoter 1B deletion leading to classical FAP in a Bulgarian family. To the best of our knowledge, such deletion has not been reported yet.

Materials and methods

The proband, a 36-year-old man of Bulgarian origin, was referred for genetic analysis after being diagnosed with classical FAP. Thousands of colonic polyps had been identified on endoscopy and full colectomy with ileo-anal reconstruction had been performed 2 years prior to the genetic referral. His father, a 65-year-old man, had had full colectomy 30 years ago because of FAP. The proband’s grandfather had died from metastatic colon cancer in his late 40 years, but no medical records were available. Thus, fresh tumour tissue was not available for genetic testing. No extra-colonic manifestations were identified in this family. Two healthy male cousins of the proband (sons of his deceased aunt), aged 46 and 49, with no polyps on endoscopies, were also genetically tested. Because the family was rather small, no other first degree relatives were available for analysis.

The study was approved by the Ethics Committee of Sofia Medical University. Written informed consent was obtained from the patients prior to genetic testing.

Genomic DNA was isolated from peripheral blood and screening for germline mutations was performed by direct sequencing (10). Multiplex ligation-dependent probe amplification (MLPA) was used for detection of larger deletions and duplications. The MLPA APC kits were obtained from MRC-Holland (Salsa MLPA probemix P043-B1 and PO43-C1 APC, Amsterdam, the Netherlands) and analysis was performed according to the manufacturer’s instructions. The polymerase chain reaction (PCR) product ratios were calculated by dividing each measured peak area by the sum of all peak areas for the particular sample. The ratio of each peak’s individual relative probe area was normalized to the mean obtained with five control samples.

Array CGH analysis was performed using custom designed whole-genome oligonucleotide arrays (OGT, UK), covering the human genome at a median density of 2.5 kb. Labelling, hybridization and washing were performed according to the manufacturer’s recommendations. Data were analysed using the CYTOSURE INTERPRET software v3.4.3. (OGT, Oxford, UK). APC breakpoints were detected by long-range PCR using primers (available upon request), located 5’ and 3’ to the putative deletion. DNA alignments were carried out according to the University of California, Santa Cruz reference sequence March 2006 (National Center for Biotechnology Information/hg18).

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Allele-specific expression (ASE) assay was performed as follows: total RNA from fresh blood samples was prepared using Trizol reagent (Invitrogen Life Technologies, Bulgaria), and cDNA was prepared with a First Strand cDNA Synthesis Kit (Thermo Fisher Scientific, Bulgaria). A 300-bp region around single nucleotide polymorphism (SNP) in c.4479G＞A, codon 1493 ACG＞ACA, found to be heterozygous in both of our patients was amplified for genomic and cDNA. After direct sequencing, ASE ratios were calculated as the ratio between the two allele’s peak area of cDNA divided by the same ratio for genomic DNA. In order to confirm consistency with ASE data, the total expression level of APC gene was measured by real-time qPCR analysis. To avoid unspecific DNA amplification, the forward primer was specific for coding exon 3 and the reverse was specific for coding exon 5 (available upon request). The relative abundance of APC gene was normalized by the expression level of the GAPDH. Observed C_T levels of patients and controls were then used to calculate fold change using the 2^−△△Ct method of relative quantification (11). All real-time qPCR analyses were performed in triplicates.

The detected APC deletion was compared with known aberrations listed in publically available databases, such as: ENSEMBL (http://www.ensembl.org website) and the Colon Cancer Gene Variant Database (http://www.LOVD.nl/APC). Repeat-masker was used to identify repetitive elements around the breakpoint region (http://www-repeatmasker.org/cgi-bin/WEBRepeatMasker, version 4.0.0).

Results

Initially, we performed direct sequencing of the proband’s DNA, covering the exons and exon–intron boundaries of the entire APC gene, but no causative DNA changes were identified. As a next step, we performed MLPA testing (P043-B1). No differences were observed between the MLPA probe peak areas of the proband and the five control samples, and we reported the family as mutation negative. After the publication of Rohlin et al., describing a partial deletion of promoter 1B, two new probes were included in the MLPA kit (MRC-Holland, The Netherlands) (P043-C1), covering the full region of promoter 1B. The first probe is located in the deleted region, described by Rohlin et al. and the second one is located just outside this deletion. We decided to re-analyse six previously tested mutation-negative FAP families, referred to our laboratory. Three of the families were presented with the attenuated form of FAP (AFAP), one case had no family history of FAP and two of the families were with classical FAP. In the proband, described in this study, we found 50% peak area decrease in two of the probes, corresponding to heterozygous deletion of promoter 1B (Fig. 1). Same result was detected with MLPA analysis of the proband’s father DNA, but not in his two healthy cousins and the five controls samples. Normal MLPA profile was also observed for the remaining five mutation-negative families.
In order to determine the boundaries of the deletion, we conducted a whole-genome oligonucleotide array. As shown in Figure 2, consistently with MLPA data, a heterozygous deletion was found in the region of 5q22.2, corresponding to a sequence upstream of the APC gene and encompassing promoter 1B. As a next step we used long-range PCR for precise mapping of the deletion junction fragments. The PCR reaction of the control DNAs failed to amplify any detectable PCR product (theoretically about 23,000 bp), whereas PCR products of \( \approx 1000 \) bp were detected from the patients. The exact breakpoints were identified by direct sequencing of the obtained PCR products – chr5: g.112,061,394_112,083,285del 21,890 insTTGCTCTATGACCAATT. The analysis of the junction sequence showed an insertion of at least 15 bp template DNA. On the basis of a recently proposed model of synthesis-dependent microhomology-mediated end joining (SD-MMEJ) (12), we assumed an insertion of 17 bp template with 2 bp flanking sequence on both sides of the insertion. The inserted DNA template was found to be located 7 bp upstream the breakpoint (Fig. 2). Real-time qPCR data indicated a decrease in expression level \( 0.221 \pm 0.14 \) [expression level \( \pm \) standard deviation (SD)] in the proband and a decrease \( 0.568 \pm 0.11 \) in his father. The total APC expression levels with the corresponding SD values are presented on Figure 3. ASE analysis was in agreement with these findings, showing about 70% decrease of deleterious allele plot peak in both of the patients (Fig. 3).
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Fig. 2. (a) Detection by genomic CGH array of a heterozygous deletion (blue box) on chromosome 5q22.2. The minimal breakpoints are defined between 112,061,819 and 112,082,393 (deletion of about 0.021 Mb) and the maximal breakpoints between 112,059,046 and 112,085,820 (deletion of about 0.027 Mb). (b) Direct sequencing of the breakpoint region. The 17 bp insertion is black boxed. The inserted template DNA sequence is underlined. Human genome reference sequence is presented. Letters in black colour are corresponding to the region up- and downstream the breakpoints, in italic are shown the putative flanking sequences. Letters in grey are corresponding to the deleted sequence.

Discussion

The mutation detection rate in FAP families varies widely – from 30% to 90%. The highest rates of detection are reported in classical FAP and the lowest in AFAP. There are some possible explanations for such variability – the presence of still unknown genes and mechanisms, involved in the aetiology of the disorder and natural flaws in the molecular genetic techniques, used for mutation screening. The use of direct sequencing, followed by MLPA assay, generally increases the mutation detection rate by 7–10%. However, many cases remain mutation negative, suggesting the putative importance of the regulatory and deep intron sequences (7).

In this report, we describe a large novel APC deletion, encompassing the full promoter 1B region. This deletion was found by the new version of MLPA in only one of the six re-analysed families. The deletion was found in one of the two families presented with the classical FAP. The family, described by Rohlin et al. was also with classical FAP (9). This observation is interesting, but due to the small sample size it would not be appropriate to speculate about genotype-phenotype correlation.

The bioinformatic analysis of the sequences around the breakpoint showed an AluSx repeat starting 150 bp downstream from the distal junction site. No repetitive elements were found at the proximal breakpoint. Alu elements are a family of short interspersed nuclear elements (SINE), presenting about 10% of the mass of the human genome and are frequently involved in rearrangements known as non-homologous end joining (NHEJ) (13). In our family, NHEJ was assumed as a mechanism of repair of the double-strand break (DSB) as there was little homology between the breakpoint flanking sequences. The insertion of a template sequence at the junction site is supporting this assumption.

We confirmed the decrease of APC expression levels by two different approaches. Both of them aimed to analyse the total expression level, generated from the two gene promoters (1A and 1B). The ASE
Fig. 3. Total adenomatous polyposis coli (APC) expression in patients and normal controls as measured by quantitative polymerase chain reaction (qPCR) and allele-specific expression analysis (a) qPCR data. The error bars indicate the standard deviation for the real-time polymerase chain reaction (PCR) replicates. Samples C1–C5 are normal controls, P1 and P2 are the proband and his father (affected by the APC promoter 1B deletion), P3–P4 are unaffected cousins of the proband. (b) Proband’s cDNA for the single nucleotide polymorphism (SNP) c.4479G>A-A 100%, G 30% (the A allele is normal and the G allele shows reduced peak height). (c) Proband’s genomic DNA-A 100%, G 100%. The site of the SNP c.4479G>A is indicated by arrow.

assay demonstrated that nearly 30% of the deleterious allele G was still present. Recently, Rohlin et al. demonstrated that the expression of promoter 1B is significantly higher compared with promoter 1A in numerous normal tissues. In addition, they reported just 8% deleterious allele expression in blood of one Swedish patient with partial promoter 1B deletion, suggesting significant functional relevance (9). In contrast, Charames et al. demonstrated complete silencing of deleterious allele transcription in one Manitoba Mennonite patient affected by promoter 1A deletion (8). Our findings demonstrated reduced total APC expression in both patients, however, remaining at levels significantly higher compared with Rohlin data. Thus, it remains unclear which promoter (if any) has dominant role in human peripheral blood. There are several possible explanations for the above discrepancies – the small number of studied patients, phenotypic variations and the concept that different mutations may cause different levels of expression even when affecting the same genomic region. We speculate that the intact promoter 1A might have significantly compensated the total APC gene blood expression in our patients, which is a matter of further study with promoter 1B transcript-specific gene expression assay.

In the light of our findings of a potentially causative APC promoter 1B deletion in one of six previously reported as mutation-negative families, it seems reasonable to consider targeted genetic screening of the APC promoter 1B region in a larger cohort of unsolved cases to evaluate the frequency and clinical relevance of deletions and point mutations.

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References