

# First genetic analysis in Tunisian familial adenomatous polyposis probands

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**Abstract.** Familial adenomatous polyposis (FAP) is an autosomal dominant inherited disease characterized by the development of hundreds to thousands of adenomatous polyps in colon and rectum. The APC gene (*adenomatous polyposis coli*) is considered as the major mutated gene in FAP. It has been shown that biallelic germline mutations in the base-excision-repair gene MYH can be responsible for a recessive inheritance of adenomatous polyposis (AP). This study is the first Tunisian genetic analysis on AP patients. Multiplex ligation-dependent probe amplification (MLPA) was used to screen the APC gene for large genomic rearrangements. The total APC and MYH exon sequences and exon-intron edges were sequenced in an effort to detect germline mutations, four were explored. Mutations were detected in four patients that fulfil the clinical criteria of AP. Three mutations were found in the APC gene, of which two were novel (c.1636\_1639delAGTG and c.2514 G>T) and all gave rise to a truncated APC protein. The missense G382D mutation, already described in north and south European populations was found in the MYH gene at the homozygous state in the fourth patient with moderate AP. Our preliminary study provides a basis for implementation of genetic counselling for AP.

## Introduction

Colorectal cancer (CRC) is the first cause of digestive cancer mortality in Tunisia. Familial adenomatous polyposis (FAP)

is one of the hereditary colorectal cancers, and represent 1% of all cases of CRC (1). FAP is an autosomal dominant disease that affects about 1/10000 individuals. Symptoms of FAP include abdominal pain, diarrhoea, haematochezia and mucus discharge but many patients are also asymptomatic. Patients with FAP develop hundreds to thousands of colon and rectum polyps (2) that may transform to cancer before the age of 40 if left untreated (3). Extracolorectal manifestations of FAP are common and characterise the Gardner syndrome (4), they include adenomas, osteomas, desmoid tumours and congenital hypertrophy of the retinal pigment epithelium (CHRPE) (5).

FAP is caused by mutations of the *adenomatous polyposis coli* (APC) gene located on human chromosome region 5q21-22 (6). The APC gene is composed of 15 exons, and germline mutations have been described on the whole APC gene, but ~65% of mutations are located in exon 15 that occupies >75% of the whole coding region (7). Mutations in codon 1309 and 1061 (deletion of 5 bp) accounts respectively for 10% and 5% of all mutations described (7,8).

The APC gene encodes a 311-kDa multi-domain protein (the APC protein) involved in the Wnt signalling pathway (the wingless signal transduction pathway) (9). This protein binds to  $\beta$ -catenin and regulates the proliferation, migration and differentiation of the normal colorectal epithelial cells (10), where it acts as a 'gatekeeper' to prevent development of tumors. It was shown that APC plays a role in apoptosis, cellular adhesion and chromosome segregation (11).

Sieber *et al* (12) described a form of adenomatous polyposis (AP) without mutation of the APC gene and characterized by a lower number of polyps, typically <100. This form of AP shows a recessive mode of inheritance in families. Patients carry biallelic germline mutations in the base-excision-repair (BER) gene MYH (MutYH), which encodes the DNA repair enzyme MYH glycosylase that is involved in the BER pathway, and conferring the term MYH associated polyposis to this particular form of AP (13). MYH protein removes mismatches 8-oxoG:A that occurs in DNA, preventing fixation of G/T transversions. In case of biallelic inactivation

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of the MYH gene, DNA mutations can accumulate and cancer may develop (13,14).

In Tunisia, FAP patients are diagnosed on the basis of family history, clinical and histological profile, but no genetic tests are available. The aim of this first Tunisian genetic study of FAP, was to analyze the entire coding region of the APC and MYH gene in four unrelated FAP Tunisian patients. This study enables the clinical spectrum of FAP in Tunisian patients to be assigned to the APC and MYH genes.

## Materials and methods

**Patients.** This study includes 4 probands from unrelated families who came for counselling at Charles Nicolle Hospital of Tunis, because of a family history of colorectal cancer or unbearable symptom. After confirmation of the FAP state by colonoscopy or by suggestive history of hereditary CRC of polyposis type, consent for the genetic study was obtained from patients, the pedigree of the family was made and 10 ml of peripheral blood was drawn in EDTA from each patient.

**DNA isolation.** Genomic DNA was purified from peripheral blood leukocytes using the QIAamp DNA blood mini kit (Qiagen; Valencia, CA, USA) following the manufacturer's instructions. A NanoDrop (ND-1000) spectrophotometer (Wilmington, DE, USA) was used to quantify the DNA.

**Multiplex ligation-dependent probe amplification (MLPA).** The Salsa MLPA P043 APC probemix kit (MRC-Holland, The Netherlands) was used according to the manufacturer's instructions to screen large deletions/duplications of one or more exons of the APC gene. In addition to probes for each exon, this kit contains one probe for alternative APC exon 10A and two probes for the wild-type sequences at mutation hot-spots 1061 and 1309, allowing direct detection of small deletions at these locations. Fragment analysis of multiplex PCR was carried out on the ABI-3730 DNA analyser (Applied Biosystems, Foster City, CA, USA), using GeneMapper software, version 4.0 (Applied Biosystems). Deletions or duplications of probe recognition sequences are detected by a 35-50% reduced or increased relative peak area of the amplification product of that probe (15).

**PCR amplification.** All APC and MYH exons were amplified by polymerase chain reaction (PCR) in a total volume of 30  $\mu$ l

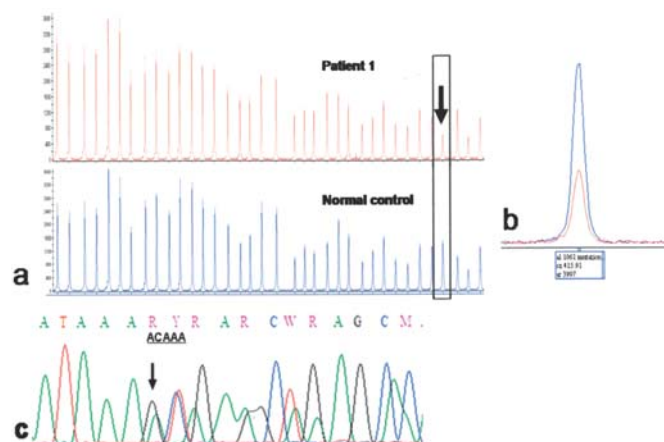


Figure 1. Detection of APC genomic deletion on patient 1 by multiplex ligation-dependent probe amplification (MLPA) and by sequencing. (a and b) APC normal control versus APC deletion in codon 1061 in patient 1, screened by MLPA (the arrow shows the differences in the 1061 peak between control and patient 1), (c) Result of sequencing analyses: deletion of ACAA in one allele in patient 1 (the arrow shows the position of the deletion).

of reaction mixture containing 100 ng of genomic DNA of each sample, 1X PCR buffer, 2.5 mM MgCl<sub>2</sub>, 10 mM dNTP, 3.3  $\mu$ M of each primers designed with primer3 software ([frodo.wi.mit.edu/cgi-bin/primer3/primer3\\_www.cgi](http://frodo.wi.mit.edu/cgi-bin/primer3/primer3_www.cgi)) (Tables I and II) and 1 U Ampli Taq Gold® DNA polymerase (Applied Biosystems). Amplification was performed on a GeneAmp PCR system 9700 (Applied Biosystems). The PCR products were analysed on an ethidium bromide stained agarose gel. Before sequencing, the positive reactions were cleaned up on a MultiScreen™ PCR 96-well plate (Millipore, Billerica, MA, USA).

**Sequencing of genomic DNA.** Sequencing reactions were carried out in forward and reverse orientations using the BigDye terminator v1.1 cycle sequencing kit (Applied Biosystems). The products of the sequencing reactions are cleaned up using the Sephadex™ G-50 (GE Healthcare, Life Sciences) in a MultiScreen®-HV 96-well filter plate (Millipore), then run up on an ABI-3730 DNA sequencer. The resulting sequence data were analyzed with the SeqScape software, version 2.5 (Applied Biosystems) in comparison with the references sequences of the APC and

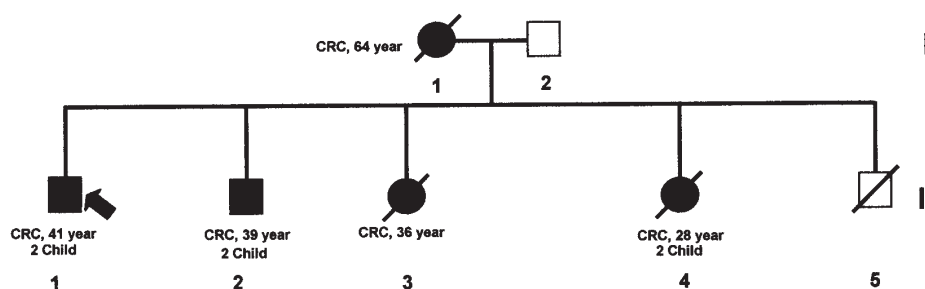


Figure 2. Family pedigree of patient 2. I-1, the patient's mother who had died of colorectal cancer at the age of 64; II-1, proband (patient 2); II-2, the patient's brother who had diagnosed with colorectal cancer at the age of 39; II-3, the patient's sister who had died of colorectal cancer at the age of 36; II-4, the patient's sister who had died of colorectal cancer at the age of 28. The arrow shows patient 2.

**SPANDIDOS** Primers and conditions for APC gene amplification by PCR.

Exon	Forward primer	Reverse primer	Tm (°C)	Fragment length (bp)
1	TTCTTTAAAAACAAGCAGCCACT	AAATGCTAACTTTTGCAAGAAAGA	58	350
2	CAAGAAATACAGAATCATGTCTTGAA	ACCAACACCCAAATCGAGAG	58	250
3	TGTTTTCAGTCATGTATATTTGTGG	TGGAGTACACAAGGCAATGTT	58	350
4	TTGCTCTTCTGCAGTCTTTATT	TCAGGCCTAAAGTTGGGTAA	48	300
5	TACAAGATATTGATACTTTTTTA	TGTAATTCATTTTATTCCTAATAGCTC	48	210
6	TGATTTGACATAACCCCTGAGC	GAACATCTATATTTCAATGGTGTCA	58	270
7	AAGAAAGCCTACACCATTTTT	GATCATTCTTAGAACCATCTTGC	50	220
8	TCATACAGACACTTCATTGGA	CAAAATGCTGGGATTACAGG	54	270
9	AGTCGTAATTTTGTCTTCTAACTC	GCTTTGAAACATGCACTACGAT	58	440
10	ACAAAGCATTATGGTTTATGTTGA	TACCATGATTTAAAAATCCACCAG	50	170
11	GATGATTGTCTTTTTCCTCTTGC	CTGAGCTATCTTAAGAAATACATG	50	200
12	CCAAAAATAAAGCTTGGCTTC	GGTTGCAGTGAGCTGAGATT	50	240
13	TTTCTATTCTTACTGCTAGCATT	ATACACAGGTAAGAAATTAGGA	50	280
14	TAGATGACCCATATTCTGTTTC	CAATTAGGTCTTTTGAGAGTA	58	290
15AB	GTTACTGCATACACATTGTGAC	TTGCTGGATGGTAGTTGCCT	58	685
15CD	ATTTGAATACTACAGTGTTACCC	TGTTTGGGTCTTGCCCATGTT	48	724
15EF	AGTCTTAAATATTCAGATGAGCAG	AGCTGATGACAAAGATGATAATG	60	804
15GH	AAGAAACAATACAGACTTATTGTG	TCCATCTGGAGTACTTTCTGTG	50	770
15IJ	AGTAAATGCTGCAGTTCAGAGG	TGAGCCTCATCTGTACTTCTGC	60	725
15K	ATCTAACAATCGAATCCCCT	TTGTGGTATAGGTTTTACTGGTG	54	350
15L	ACCCAACAAAAATCAGTTAGATG	GTGGCTGGTAACTTTAGCCTC	60	400
15M	ATGATGTTGACCTTTCCAGGG	ATTCTGTAACTTTTCATCAGTTGC	58	230
15N	AAAGACATACCAGACAGAGGG	CTTTTTTGGCATTGCGGAGCT	60	330
15O	AAGATGACCTGTTGCAGGAATG	GAATCAGACGAAGCTTGTCTAGAT	60	270
15P	CCATAGTAAGTAGTTTACATCAAG	CTTAAGGGGTGGGCCTTTT	54	230
15Q	CAGCCCCTTCAAGCAAACATG	GAGGACTTATTCCATTTCTACC	60	360
15R	CAGTCTCCTGGCCGAAACTC	GTTGACTGGCGTACTAATACAG	60	350
15S	TGGTAATGGAGCCAATAAAAAGG	TGGGAGTTTTCGCCATCCAC	58	300
15T	TGTCTCTATCCACACATTCGTC	ATGTTTTTCATCCTCACTTTTTGC	58	280
15U	TTCCTCGAGTAAGCACTTGGA	TTGAATCTTTAATGTTTGGATTTGC	58	420
15V	TCTCCACAGGTAATACTCCC	GCTAGAACTGAATGGGGTACG	60	260
15W	CAGGACAAAATAATCCTGTCCC	ATTTTCTTAGTTTCATTCTTCCTC	50	330

MYH gene (Accession number respectively; NM000038, NM\_012222) downloaded from the NCBI Genbank website (<http://www.ncbi.nlm.nih.gov/>). All mutations were verified in both forward and reverse directions using PCR products from independent reactions.

## Results

In this study, the genetic analysis of FAP was carried out on four unrelated Tunisian patients. Their age ranged between 13 and 43 years (3 males, 1 female). All displayed colorectal polyps, three had colon cancer and one underwent total colectomy. No extracolorectal manifestations were found in any of the patients. Our approach was to screen the APC gene by the MLPA technique to detect the possible presence

of large genomic rearrangement (deletion, duplication) and to sequence the exons and intron-exon boundaries of the APC and MYH genes.

Patient 1 is a 35-year-old woman who presented with classical symptoms of FAP (>1000 polyps) and was diagnosed with colon cancer. MLPA analysis (Fig. 1a and b) revealed a known heterozygous mutation (7,11) on codon 1061 of exon 15 in the APC gene (c.3183\_3187 delACAAA). This 5-bp deletion introduces a frame-shift mutation and a stop codon (p.Lys1061fsX1062) and was confirmed by sequencing (Fig. 1c). Patient 2 is a 41-year-old man that had contacted the Charles Nicolle Hospital of Tunis because of his concern of a family history of colon cancer. His mother and his two sisters had died of CRC. His brother was also affected with CRC (Fig. 2). On colonoscopy, he was found to

Table II. Primers and conditions for MYH gene amplification by PCR.

Exon	Forward primer	Reverse primer	Tm (°C)	Fragment length (bp)
1	CTTCCCCTCTCCCAGAGC	GACCCGGGACGTCTGAAC	60	254
2	CAAGAGTAAACCCGTGAGCA	AGCCTGAATCTGCCTTTCAT	59	329
3	AAGCCCTAAGTGGGAGCATA	CAGATGAGGAGTTAGGGTGGAA	55	323
4	ACCATGGAGAAGACGGGTAG	ATGAGGACACTGCTGACCTG	62	239
5	CTGAGAGCAGGGCCACTT	GGGTGAAGGTGGTAGAGGAA	60	276
6	GGTCAGACCCCAGATGAGAG	CCCGTCAGTCCCTCTATTGT	63	200
7-8	GGA CTGACGGGTGATCTCTT	CAGAGGGGCCAAAGAGTTAG	62	382
9	CATTGCCTCTATCGCCTTTG	GAAGGGAACACTGCTGTGAA	60	282
10-11	AGGATGTTGGGGTAACAAGG	AGAACTGGAATGGGGCTTCT	60	432
12	TCTCTAGGTTGGCCCCTAAA	CGATTCCCTCCATTCTCTCT	60	308
13	GGCAGTGGCATGAGTAACAA	CCTCAAAAGCCAACATCCTT	59	260
14	CACCTTGGGGAGGTAAGTGA	GGAAACACAAGGAAGTACAACAAA	57	291
15	GGACATGAAGTTAAGGGCAGA	AACTATTCCTCCCTCCAGTGAA	51	250
16	CTACAAGGCCTCCCTCCTTC	ACATAGCGAGACCCCATCT	63	269

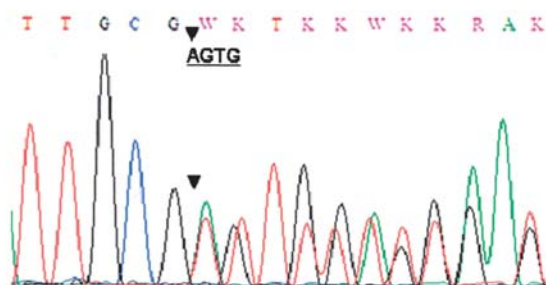


Figure 3. Detection of APC genomic deletion on patient 2 by sequencing. Deletion of AGTG in one allele at codon 546 (the arrows show the position of the deletion).

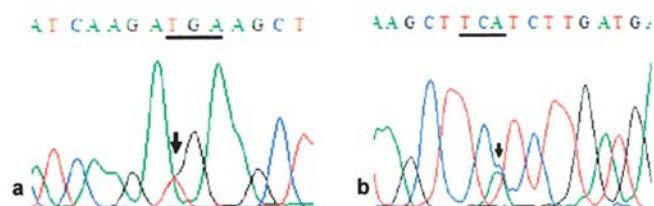


Figure 4. Detection of APC genomic transversion on patient 3 by sequencing. G to T transversion at the codon 839 (GGA → TGA), resulting in a stop codon. (a) Forward sequence, (b) Reverse sequence (the arrows show the position of the substitution).

have a sigmoid adenocarcinoma and hundreds of polyps spread in the transverse and ascendant colon. He underwent a total colectomy. A heterozygous germline mutation was diagnosed for this patient in exon 13 of the APC gene by sequencing (Fig. 3). There was a frame shift mutation at codon 546 consisting of a four-nucleotide deletion (c.1636\_1639delAGTG), resulting in a stop codon at position

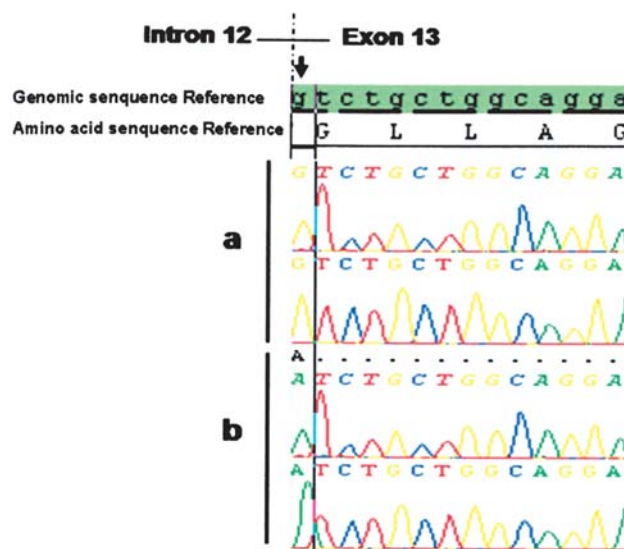


Figure 5. Alignment with the SeqScape software of two genomic MYH sequences showing a variation in the initial codon of exon 13. (a) Normal sequence with the guanine nucleotide at the 1145 position, (b) variant sequence of patient 4 with the homozygote adenosine nucleotide at the 1145 position.

547 (p.Ser546fs X 547). Since this mutation is absent of the APC database (7,11,16) it appears that it is a novel germline mutation. Patient 3 is a 13-year-old child; his mother brought him to the hospital because his father and uncle had died of colorectal cancer at the fourth decade of their life and had presented major symptoms of FAP. The child refused colonoscopy. A novel germline mutation was detected and identified in the APC gene of this patient (7,11,16). It is a heterozygous G to T transversion at codon 839 (GGA → TGA; c.2515 G>T), resulting in a stop codon (p.Gly839X) on one allele of the APC gene (Fig. 4). Patient 4 is a 43-year-old man who had an examination at the gastro-enterology

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cause of digestive pain; his colo-noscopy showed a number of polyps (eight tubular adenomas). No mutation was found in the APC gene either using the MLPA test or by the total sequencing of the APC gene. The analysis of the total MYH gene by sequencing showed an already characterized mutation (17,18) in the first codon of exon 13 (Fig. 5), it is a G to A transition (c.1145 G>A; p.Gly382Asp) for which the patient is homozygous.

## Discussion

This genetic study is the first mutation analysis of AP in Tunisia. We found a germline mutation in the four patients with clinical criteria of AP that were explored at the molecular level. We performed MLPA analysis and sequencing of the APC and MYH genes (exons and exons-intron boundaries) to detect mutations. We detected two novel germline mutations (Figs. 3 and 4) that are not mentioned in the APC data base (7,11,16). These two mutations are located respectively at codon 546 and 839, and result in premature stop codons. A third truncating mutation was detected in exon 15 of the APC gene at codon 1061 using the MLPA technique (Fig. 1a and b). This known mutation is recurrent and is also frequent in other populations (7,11,16,19,20). The APC protein is an integral part of the *wnt*-signaling mechanism; it interacts with  $\beta$ -catenin and regulates its activity. The abolition of this interaction causes the accumulation of  $\beta$ -catenin protein in the cytoplasm of the colorectal cells thus promoting the generation of tumors (21,22).

Investigation of the MYH gene showed for one patient (Fig. 5), a homozygous G382D germline missense mutation which has been reported as the most common deleterious mutation of the MYH gene in the Caucasian population (12,18,14,23). In this case, the recessive phenotype of the disease is confirmed in this particular form of FAP linked to MYH mutation (13,14). The effect of this MYH variant was studied by Al-Tassan *et al* (18) who showed that the mutated MYH protein expressed in *Escherichia coli* results in severe impairment of base excision repair. Subsequently, the biochemical function of the human G382 MYH protein was also shown to be severely compromised (24-26).

Our results are also important for genetic counselling and further efficient treatment and care of mutation carriers. Mutation identification can differentiate between the 50% risk of disease for progeny as observed for FAP with APC mutation, and the particular risk associated with consanguinity in the case of the recessive mode of disease inheritance for MAP. This study brings noteworthy new data concerning susceptibility to colon cancer due to FAP. It shows that even studying a restricted number of patients, we can confirm that the same genes are at play in the Tunisian population, including the APC and the more recently identified MYH gene. This has significant implications for genetic counselling in Tunisia, showing the importance of testing these two genes. Since consanguinity is a demographic characteristic in Tunisia, favouring the occurrence of recessive genetic diseases it is likely that the MYH gene is at play in a high proportion of AP. The mutation we found in the MYH gene is also a recurrent mutation in Caucasian population

from north to south Europe, suggesting that it is a very ancient mutation (18).

A large molecular study of Tunisian patients should be undertaken, based on the presence of consanguinity and of early diagnosed colon tumors with clinical presentation compatible with MYH germline mutation in family members. Identification of MYH mutations would enable further genetic counselling and screening programs. Our study also confirms the heterogeneity of APC mutation by describing two new mutations of the APC gene in two of our patients. This clearly indicates that a large spectrum of mutations will be found in Tunisian patients.

From a public health point of view, and regarding economical constraints, our study clearly shows that stringent clinical criteria can yield a high proportion of positive molecular genetic results, which can further benefit rational screening and prevention.

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