European Journal of Human Genetics (2002) 10, 505–510

www.nature.com/ejhg

ARTICLE

Pathogenic mutations and rare variants of the *APC* gene identified in 75 Belgian patients with familial adenomatous polyposis by fluorescent enzymatic mutation detection (EMD)

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Familial adenomatous polyposis (FAP) is a dominant inherited colorectal cancer syndrome which is caused by germline mutations in the *adenomatous polyposis coli (APC)* gene. Enzymatic mutation detection (EMD) has potential advantages over the standard protein truncation test (PTT) that is currently used in screening the *APC* gene for mutations. First we wanted to validate the EMD technique in comparison to PTT. Secondly, we wanted to develop an efficient working protocol for EMD screening of *APC*. Seventy-five unrelated patients were screened for mutations. All mutations that had previously been detected by PTT were also identified by EMD; the sizes of the cleavage fragments were as expected according to the position of the mutations within the amplicons. A new screening strategy based on EMD allows the analysis of the *APC* gene in 31 overlapping PCR fragments. In total, EMD efficiently detected the 26 truncating mutations in this series. In addition, two rare variants were also detected: the first is the typical Ashkenazi missense mutation I1307K while the second variant, E1317Q, has been identifed in Belgian patients and controls, and should no longer be considered as a pathogenic mutation, but rather classified as a polymorphism.
European Journal of Human Genetics (2002) **10,** 505–510. doi:10.1038/sj.ejhg.5200825

Keywords: enzymatic mutation detection; EMD; variants; hypermutable; FAP; *adenomatous polyposis coli* (*APC*); protein truncation test

Introduction

Familial adenomatous polyposis (FAP) is an autosomal dominant disorder characterised by the development of at least 100 colorectal adenomas at a young age (before 20 years), some of which will progress to colorectal cancer one or two decades later. In addition to the classical form of FAP, an attenuated form was described: attenuated FAP (AFAP) or attenuated adenomatous polyposis coli (AAPC), in which the number of polyps ranges between 10 and 99, appearing at a later age and being still associated with an increased risk of cancer.[1] The profuse phenotype is associated with the appearance of more than five thousand polyps, whereas patients with the classical sparse phenotype eventually develop one to two thousand polyps. The *adenomatous polyposis coli* (*APC*) gene was discovered in 1991,[2,3] and the germline mutation spectrum is generally spread over its entire coding region. Hot spot regions for somatic mutations in colorectal tumors (mutation cluster region (MCR) in exon 15),[4] and in desmoids (middle part of exon 15)[5] are well characterised. The positions of the germline mutations correlate with the severity of the disease. For example, in classical FAP, mutations are located between

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Received 23 July 2001; revised 18 April 2002; accepted 23 April 2002

codons 213–1249, whereas in the profuse phenotype mutations are located between codons 1250–1464.[6] Mutations in exon 9 and at the 5′ and the 3′ end of *APC* are also responsible for AFAP.[7–9] Known germline and somatic mutations can be found at the internet site address: http://perso.curie.fr/Thierry.Soussi/APC.html#Ancrage3.

Due to the extended mutation spectrum of the *APC* gene, and the size of the gene itself, there is a need for an efficient and sensitive mutation detection method. Currently the protein truncation test (PTT) is the technique most widely used to screen for mutations in the *APC* gene.[10,11] However, PTT only allows the detection of truncating mutations. While over 90% of pathogenic mutations detected in classic FAP are truncating mutations, missense mutations, polymorphisms and variants of unknown significance that have been reported are spread over the whole gene. The majority of these would not have been detected by PTT (e.g. E1317Q, S1971R, A2119V).[12,13]

We wanted to evaluate the feasibility and efficiency of enzymatic mutation detection (EMD) screening of the *APC* gene. This is potentially an easy, rapid and accurate method that has the advantage of detecting all types of mutations.[14] EMD is based on the properties of the T4 endonuclease VII that has the ability to detect mismatches in a double-stranded DNA molecule and to cleave the DNA strand at this locus.[15] The cleavage products can easily be detected after electrophoresis. EMD has been originally developed from the enzymatic mismatch cleavage method (EMC),[16] and has been improved to allow extended screening.

In this study, we aimed to validate EMD by comparing it to PTT. In addition, we established an efficacious screening protocol which allows rapid scanning of all exons of *APC*.

Subjects and methods

DNA from clinically affected FAP or AFAP patients submitted for molecular diagnostic analysis was used for this study. Lymphocyte DNA from patients and controls was prepared using standard salt precipitations.

For EMD analysis of exon 15, amplicons of different sizes were generated using several combinations of described primers.[2] In addition, a few new primers were designed: c3 (forward) 5′-CACAAGCAAAGTCTCTATGGTG, h2 (reverse) 5′-TTTCTGCCTCTTTCTCTTGG, i3 (forward) 5′-ACCAAGAGAAAGAGGCAGAA. The amplicons were: a-c, c3-d, d-e, f-f, g-h, h-h2, i3-i, j-k, k-l, m-n, o-p, q-r, s-t, u-v, and v-w. The remaining 16 segments of *APC* (1–9, 10, 11–14, and the alternative spliced exons 9A and 10A) were amplified as described.[2,17] For each amplicon, both forward and reverse primers were labelled with fluorescein isothiocyanate (FITC). Fluorescently labelled PCR fragments were submitted to the EMD reaction with the Passport kit for ABI 377 detection (Amersham Pharmacia Biotech). Denaturation (95°C, 4 min) was followed by a cooling step from 95°C to 37°C at 0.02°C/s. Enzyme was then added to the samples and incubation for 30 min at 37°C followed. All the incubation steps were performed in a standard thermocycler. EMD products were loaded on a 6% Long Ranger Urea gel (SanverTech) and detected with an ALF DNA sequencer (Amersham Pharmacia Biotech). PTT analysis of exon 15 has been performed as described.[11]

Exon 6 has been screened for all patients using Denaturing Gradient Gel Electrophoresis (DGGE) according to Olschwang *et al*.[18]

Results

PTT was initially used for the *APC* exon 15 screening of a series of 40 patients. It has been performed in four overlapping fragments. In fifteen out of the 40 patients, a truncated peptide was detected; sequencing revealed ten different alterations that were all localized in the first half of exon 15.

For comparisons, these samples were submitted to EMD in amplicons of several sizes, ranging from 277 to 910 bp. The size of the cleavage peaks could be calculated according to the position of the mutation within the tested amplicon. As both primers were labelled, two peaks were expected for detection after cleavage. All peaks were positively detected and some mutations were observed in overlapping amplicons (Table 1).

The samples showing a negative PTT result have been further analysed by EMD of the exons 1–14. A second series of 35 patients, which had not been previously tested

Table 1 Germline mutations of the *APC* gene identified in Belgian FAP patients

Mutation	*Codon*	*Exon (fragment)*	*Detection method*	*Patient*
R216X	216	6	DGGE	187832
R232X	232	6	DGGE	191930
R302X	302	8	EMD	186794
1087 ins A	357	9	EMD	202032
S457X	457	10	EMD	179129
1494 del C	491	11	EMD	207230
R499X	499	11	EMD	151913; 178620
2128 del G	704	15-ac	EMD	110175
Y935X	935	15-ac/c3d	PTT–EMD	38378
2911 del AATA	966	15-c3d/de	PTT–EMD	30913
3104 del TT	1028	15-c3d/de	PTT–EMD	175491
3201 del ACAAA	1061	15-de	EMD	153316
Q1175X	1175	15-ff	EMD	228789
S1194X	1194	15-ff	PTT–EMD	180798
3614 ins A	1199	15-ff	PTT–EMD	35963
3945 del AAAGA	1309	15-gh	PTT–EMD	167202; 167877; 163927; 185236; 185266; 216937
4057 GC→A	1347	15-gh	PTT–EMD	193859
4411 del AG	1465	15-gh/hh2	PTT–EMD	187805
4439 del C	1474	15-gh/hh2	PTT–EMD	177751
4630 del GA	1538	15-hh2	PTT–EMD	101206

Numbering of nucleotides (nt) and codons is related to the GenBank file M74088 (nt 1 in position 1 of cDNA; codon 1 is first ATG at nucleotide 19 of cDNA).

by PTT, was also submitted to EMD according to the presented protocol (see Subjects and methods). In total, 26 pathogenic *APC* mutations were identified in 75 FAP or AFAP patients (35%). They represent twenty different DNA alterations (nine deletions, two insertions, eight nonsense mutations and one GC→A mutation) (Table 1). The mutations were located in exons 6, 8, 9, 10, 11, and in fragments *a* to *i* of exon 15. Six patients carried the same 5 bp deletion at codon 1309. This particular mutation represents nearly a quarter of all positive samples in this study. In total, six novel mutations were identified in this Belgian patient population in six different families. Table 2 describes the polymorphisms and variants identified by EMD. Clinical data for the patients harbouring new mutations are listed in Table 3.

In Figure 1 the cleavage profiles of three mutations located in amplicon c3-d of exon 15 are shown. The control sample, which is not cleaved by enzyme, gives a full-size peak of 790 bp. The heteroduplexes, present in the PCR products generated from heterozygous samples, are efficiently cleaved; two peaks can be observed for each sample. The normal and mutant homoduplexes are not cleaved and are detected as full-size peaks of lower intensity compared to the control sample.

The background level for EMD analysis of exon 6 was high, and interpretation of the results was difficult. Other conditions for hybridisation were applied (95°C, 4 min; room temperature, 15 min), but the background could not be decreased (results not shown). When PCR samples were loaded without having undergone an EMD reaction, the background was already present. This suggests that the PCR of this fragment caused the problems which we have not been able to solve. As an alternative, it was decided to screen this exon by DGGE, which enabled the detection of two nonsense mutations, R216X and R232X.

All the patients selected for this study have been screened for two variants located in exon 15 (fragment g–h): I1307K and E1317Q. EMD has been performed in two successive steps: the PCR samples were studied alone, or mixed with a negative control prior to the EMD hybridisation step, which allows the detection of heterozygotes and homozygotes respectively. One patient (234429) was found to be a carrier of the I1307K mutation; he was the only one detected in this series. Two patients (226582 and 180798) were heterozygotes for the E1317Q variant; no homozygote has been found in this series. The screening of a control population for the E1317Q allele detected one homozygous sample out of 180 controls (two alleles out of 360 or 0.6%); in this same group, no heterozygous sample has been detected by EMD (details not shown).

Discussion

In this study we present the results of the EMD mutation scanning of the *APC* gene. The first part of the study compared EMD to the standard PTT method. Six deletions,

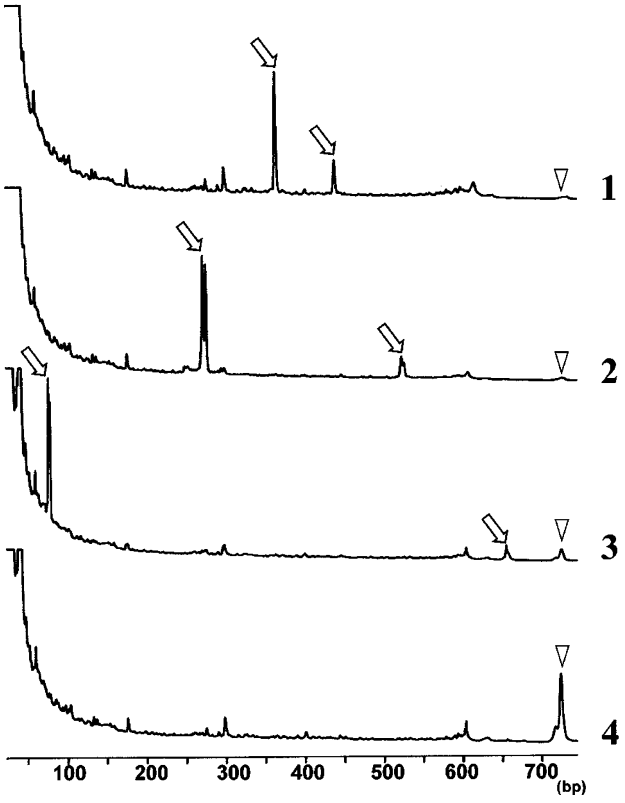

Figure 1 EMD analysis of *APC* exon 15 fragment c3-d. Cleavage profiles of three mutated samples and one control sample are shown. For PCR, forward and reverse primers labelled with FITC were used; the full-size product is 790 bp and designated by a triangle. The comparison with a control patient (lane 4) makes it possible to pinpoint the specific peaks resulting from enzymatic cleavage of the two heteroduplexes present in each PCR product. Other fragments are present both in control and patients and result in background generated by the PCR itself and/or by the enzymatic reaction. Specific peaks are indicated by arrows. Lane 1: patient 38378 bearing Y935X, expected cleavage peaks of 357 and 433 bp; lane 2: patient 30913 bearing 2911 del AATA, expected peaks of 269 and 521 bp; lane 3: patient 175491 bearing 3104 del TT, expected peaks of 76 and 714 bp; lane 4: control patient with no specific cleavage products.

Table 2 Polymorphisms, unknown variants and missense mutations identified by EMD

DNA change	*Exons*	*Mutation*	*Comment*
TAC→TAT	11	Y486Y	polymorphism
GCA→GCG	13	A545A	polymorphism
TAC→TAT	15	Y935Y	polymorphism
ATA→ATT	15	I1055I	polymorphism
ATA→AAA	15	I1307K	premutation
GAA→CAA	15	E1317Q	unknown variant
ACG→ACA	15	T1493T	polymorphism
GGA→GGG	15	G1678G	polymorphism
GAC→GTC	15	D1822V	polymorphism
CCA→CCG	15	P1960P	polymorphism

Table 3 Genotype–phenotype correlation of the novel mutations identified in this study

			Number of adenomas				
Mutation	*Age of onset*	*Colon*	*Duodenum*	*Rectum*	*Fundic cystic glands*	*Osteomas*	*Phenotype*
1494 del C	unknown	>100	n.i.	n.i.	n.i.	n.i.	classical
2128 del G	18	>100	yes	n.i.	no	no	classical
3104 del TT	10	30	yes	yes	yes	yes	classical
3614 ins A	23	>5000	no	n.i.	n.i.	n.i.	profuse
4057 GC>A	13	>100	no	yes	n.i.	n.i.	classical
4439 del C	27	>100	yes	no	yes	no	classical

n.i.: not investigated

one insertion, two substitutions, one GC→A mutation had been previously detected by PTT of exon 15. For comparison with EMD, overlapping amplicons harbouring these 10 mutations were generated. Two cleavage fragments could be observed for all samples. The method has previously been applied in a hereditary non polyposis colorectal cancer (HNPCC) study: Otway and coworkers succeeded in the radioactive detection of 91% of all mutations in the *hMLH1* and *hMSH2* genes. They could not explain why six samples did not give any positive EMD pattern; two of these mutations were insertions.[19]

In the second part, we extended the EMD screening protocol. Based on the published mutation spectrum, it was decided to screen the entire coding region of the *APC* gene (see Subjects and methods).

With the analysis of 31 amplicons of the *APC* gene by EMD, only one unexplained complication was observed for analysis of exon 6. The use of a primer, 74 bp upstream of the exon, resulted in unspecific amplification. To circumvent the problem, this exon was screened by DGGE, for which the upstream primer was located close to the intron–exon boundary in comparison to the primer used for EMD.

Thus, the EMD method faces one intrinsic problem, ie the requirement to amplify relatively ample intronic sequences to warrant detection of variants within the coding region and flanking sequences: in this study, the PCR products were not purified before enzymatic digestion and gel analysis, with the consequence that the excess of labelled primers blurred the detection at the 5′ end of the fragments. Small digested fragments (below 70 bp) are sometimes not detectable. This inconvenience can be avoided by the use of overlapping fragments (which is generally the case in the protocol presented here, and especially true for exon 15), or by the purification of PCR products prior to EMD. Alternatively, since both ends of the fragments are labelled, two cleavage peaks and thus reciprocal images are obtained, ie any abnormality will show up both at the proximal and distal end of the electropherogram.

Reported polymorphisms or variants of unknown significance are spread over the whole coding region, and they are also detected by EMD. For this study, cleavage peaks giving the same EMD profile with a frequency higher than 5% in the FAP population were not further analysed by sequencing as they were considered as frequent polymorphisms in the Belgian population. A minor drawback is that these polymorphisms could interfere with the results as EMD would not distinguish between polymorphism and a pathogenic mutation located at about the same position because both profiles would be identical. This would be limited to point mutations that affect this specific nucleotide or the flanking nucleotides. The resolution of the method is such that differences of 1 or 2 bases are discernable. In the latter case, the fragment would thus be sequenced for confirmation.

Germline mutations in the exons 4, 9, and the 3′ end of exon 15 have been associated with AFAP.[7–9,20] In patient 202032, with less than 10 polyps by the age of 62 years, a mutation was identified: an insertion at codon 357 in exon 9. This mutation has previously been described.[20] The attenuated phenotype is explained by the position of the mutation in the alternatively spliced fragment of exon 9. In patient 179129, a truncating mutation (S457X) was detected in exon 10. This patient was diagnosed at the age of 41 years with only a few polyps (<100) in colon and rectum. A sub-total colectomy has been performed, and since then clinical follow-up did not show any recurrence of adenomas (stomach normal). His phenotype can be classified as attenuated, which is rather unusual with regard to the position of the mutation. It had already been described in a FAP patient with a classical phenotype (at least 100 adenomas).[21]

Recent studies showed that apparently silent DNA changes can be responsible for a higher risk of colon cancer: the *APC* allele I1307K is known to increase the risk of transition from polyp to colorectal carcinoma in the Ashkenazi Jewish population.[22,23] As a consequence of the ATA→AAA substitution, corresponding to I1307K, an (A)8 tract is generated on the mutated allele. It has been demonstrated that this A stretch can cause polymerase slippage during replication and could act in this way as a 'premutation'.[24] Patient 234429 who was heterozygote for this mutation is of Ashkenazi Jewish decent, had 10 polyps at diagnosis by the age of 58 years, and his phenotype is

thus clearly attenuated. Our data is in agreement with the published results.

A second variant, E1317Q, has also been described as a more common *APC* variant that had been identified in 4% of AFAP patients with or without family history of colon cancer.[12,25] In our series, two patients were heterozygous for the E1317Q allele. Patient 226582 had an attenuated phenotype (10 polyps by the age of 39 years), and no other alteration was identified after EMD analysis of *APC*. The second E1317Q carrier in this FAP population was patient 180798, who was also heterozygous for the S1194X nonsense (truncating) mutation in exon 15. Further genetic analysis of this family showed that his two children, both affected, have inherited both the nonsense and the missense mutations, while their mother is homozygous for the respective wild-type alleles. The missense mutation is thus localized on the allele harbouring the nonsense mutation. Hence, the significance of the E1317Q allele remains unclear. We did not identify heterozygotes in a series of 180 controls, but identified one control who was homozygous. The E1317Q has thus an allele frequency of 0.6% in this Belgian control population. The homozygous DNA sample was from a man of 86 years of age who had no clinical history of colon cancer. This suggests that this allele is probably not pathogenic. Our data is not in agreement with the inferences made by Lamlum *et al*., who identified two carriers in 503 population-based UK controls (allele frequency of 0.2%), and seven in their series of 164 patients with multiple colorectal adenomas (3–96 tumors).[25] Four out of the seven patients had a positive family history of colon cancer. The low number of adenomas may correspond to an attenuated form, which is similar to the phenotype observed in patient 226582. Definitely, a larger survey is required to settle this problem.

Based on genotype-phenotype correlations and mutation frequency in the *APC* gene that were published worldwide, the following screening protocol could be proposed:

(1) A partial analysis of exon 15: amplicons g–h contains the frequent deletion at codon 1309 that is associated with classical (or more severe) FAP phenotype. The rare variants I1307K and E1317Q will also be detected, but the phenotypic characteristics that could be associated with these two mutations have to be studied further.
(2) For classical FAP, the second frequent deletion at codon 1061 will be picked up by analysing amplicons d–e.
(3) Attenuated forms can be associated with mutations in exons 9 and 10. Mutations in exon 4 are also frequently associated with AFAP, although no positive sample has been found in this study.
(4) Rare frameshift mutations in the second part of exon 15 have been also associated with AFAP. Analysis of the region including the amplicons k–l to v–w, is thus necessary to detect these truncating mutations.

In general, since the mutations are spread throughout the gene, one would have to complete a broader screening that also encompasses the first exons (no mutation has been found in the two first exons and in exon 10A), for routine diagnostics.

In total, 26 pathogenic mutations have been identified out of 75 FAP or AFAP patients after complete *APC* screening, and one more patient was heterozygous for the I1307K variant. This corresponds to a rather low mutation detection rate. However, among the patients with a negative result after complete screening, there is a large portion of AFAP patients with evident familial history of polyposis. This group represents 22 AFAP out of the 48 patients who were negative upon screening. Recently, identical observations have been made by Friedl and coworkers: among 680 index patients that were screened for *APC* mutations, the mutation detection rate was weaker in AFAP patients than in patients diagnosed with more than 100 adenomas (31.7% *vs* 58.2% respectively).[26] There are different possibilities to explain the limited mutation detection rate in AFAP patients: (1) The methods we used (PTT, EMD) would not have detected large genomic deletions, as they both rely on PCR amplification of genomic DNA. PTT can detect large deletions if it is carried out on RNA.[27] The use of polymorphic intragenic and extragenic markers (microsatellites or single nucleotide polymorphisms – SNP's) also allows the detection of loss of heterozygosity;[28] (2) Possibly, mutations in other genes could also be the cause of the attenuated forms of hereditary colon cancer, like the HNPCC genes (*hMLH1* and *hMSH2*), or in genes that are still unknown.

EMD is compatible with most laboratory equipment since one can choose between the use of radioactively labelled PCR products or the use of fluorescently labelled products for ALF DNA sequencers (Amersham Pharmacia Biotech) or ABI sequencers (Perkin Elmer). The latter allows the use of several dyes, which is more suitable for predicting the exact localisation of the mutation: forward and reverse primers can be labelled with different fluorophores. This also facilitates peak detection by making background signals from the two strands non-additive. We performed EMD detection on an ALF DNA sequencer, which allows the loading of 40 samples, and the use of one dye (fluorescein isothiocyanate or FITC). As both primers were labelled with the same dye, the detected peaks did not enable the prediction of exact localisation of the genetic alteration within the studied PCR product. The minor drawback of this setting is that direct sequencing of the two sites where the mutation is expected to be identified is then required.

For the Passport kit, the hands-on time is less important than for PTT, which proceeds in several incubation steps for signal enhancement and includes gel drying. The experimental time can be decreased by the use of sequencers that allow the loading of a large number of samples. High throughput can also be achieved by automation in pipet-

ting and the use of capillary sequencers that avoid preparation and handling of sequencing gels.

In conclusion, we showed that the EMD technology presented here is rapid, easy, non-toxic and avoids the use of radioactive isotopes. The screening strategy comprises an EMD scanning of large overlapping PCR fragments covering the entire coding region of *APC*. All mutations previously identified by PTT have been detected. In addition, the I1307K 'premutation' and nine polymorphisms (including the E1317Q variant) have been identified. It is therefore an elegant alternative to PTT.

Acknowledgements

We thank M Smaers (Center for Human Genetics, UCL, Brussels) and J Wijnen (Leiden University) for providing positive controls for APC mutations. We are grateful for advice in EMD mutation detection provided by Amersham Pharmacia Biotech. S Tejpar was supported by a grant of FWO – Flanders, Belgium.

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