

Short reports

Presymptomatic diagnosis in Portuguese FAP families using intragenic RFLPs and (CA)_n flanking markers by fluorescence based semiautomated DNA analysis

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Abstract

Owing to the large size of the APC gene, responsible for familial adenomatous polyposis, direct screening for individual mutations is not a practical approach. In the present study we establish the methodology of fluorescence based semiautomated DNA analysis to perform presymptomatic diagnosis of members at risk from 11 Portuguese FAP families with three (CA)_n markers flanking the APC gene, MBC, CB26, and YN5.64, and four intragenic RFLPs. Haplotypes were constructed on the basis of individual genotypes and their segregation through generations were followed.

The study was informative for 12% of subjects using only intragenic RFLPs and increased to 90% when we used the three (CA)_n flanking markers. We report two of the 11 families under study in our laboratory and show recombinant events leading to a precise localisation of the CB26 marker between D5S82 and the APC gene. In one family there was a loss of (CA) units of one allele of the CB26 marker from an unaffected mother to her son.

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Key words: familial adenomatous polyposis; presymptomatic diagnosis; fluorescence technology.

Familial adenomatous polyposis (FAP) is an autosomal dominant disorder with high penetrance and highly variable expressivity. Affected subjects develop multiple polyps in the colon and rectum, some of which become malignant unless the affected bowel is removed.

The FAP locus has been assigned to chromosome 5q by cytogenetic studies¹ and subsequently to 5q21-22 by linkage analysis.^{2,3} The gene responsible for the disease called APC (adenomatous polyposis coli) has been identified and characterised and specific germline mutations have been identified in affected subjects.⁴⁻⁷ About 200 different germline mutations in the APC gene have been described to date, most of them single base pair changes leading to termination codons or frameshift mutations that result in a downstream stop codon.⁸⁻¹³

Because the APC gene consists of 15 exons with an open reading frame larger than 8.5 kb, it is extremely difficult to screen or sequence this gene rapidly. Thus, direct screening for individual mutations in this gene is not a practical approach. However, it has been possible to perform indirect presymptomatic diagnosis or risk evaluation in the majority of cases with several highly informative microsatellite markers flanking the APC gene and some intragenic RFLPs that were recently isolated.¹⁴⁻²⁵

A genetic map with the order and physical distances of all the proximal and distal genetic markers available (on chromosome 5q) outside the immediate vicinity of the APC gene must be well defined in order to construct the haplotypes correctly and to follow their segregation through generations.

In the present study we used three (CA) repeat markers, MBC (MCC gene), YN5.64 (D5S82), and CB26 (D5S299), and to increase the accuracy of the microsatellite study we used an automated multicolour fluorescent gel scanning in a 373A Sequencer (ABI) and automated size was assigned by GENESCAN 672 software (ABI). We also studied in the same subjects four intragenic RFLPs (*Rsa*I, *Bst*HKA1, *Msp*I, *Ssp*I) described elsewhere²²⁻²⁵ and haplotypes were constructed on the basis of individual genotypes.

Blood samples were collected from all available members of 11 Portuguese FAP families with a total of 60 members. In these families affected subjects were previously diagnosed by clinical criteria in the Gastroenterology Division at the Portuguese Oncological Hospital and Egas Moniz Hospital.

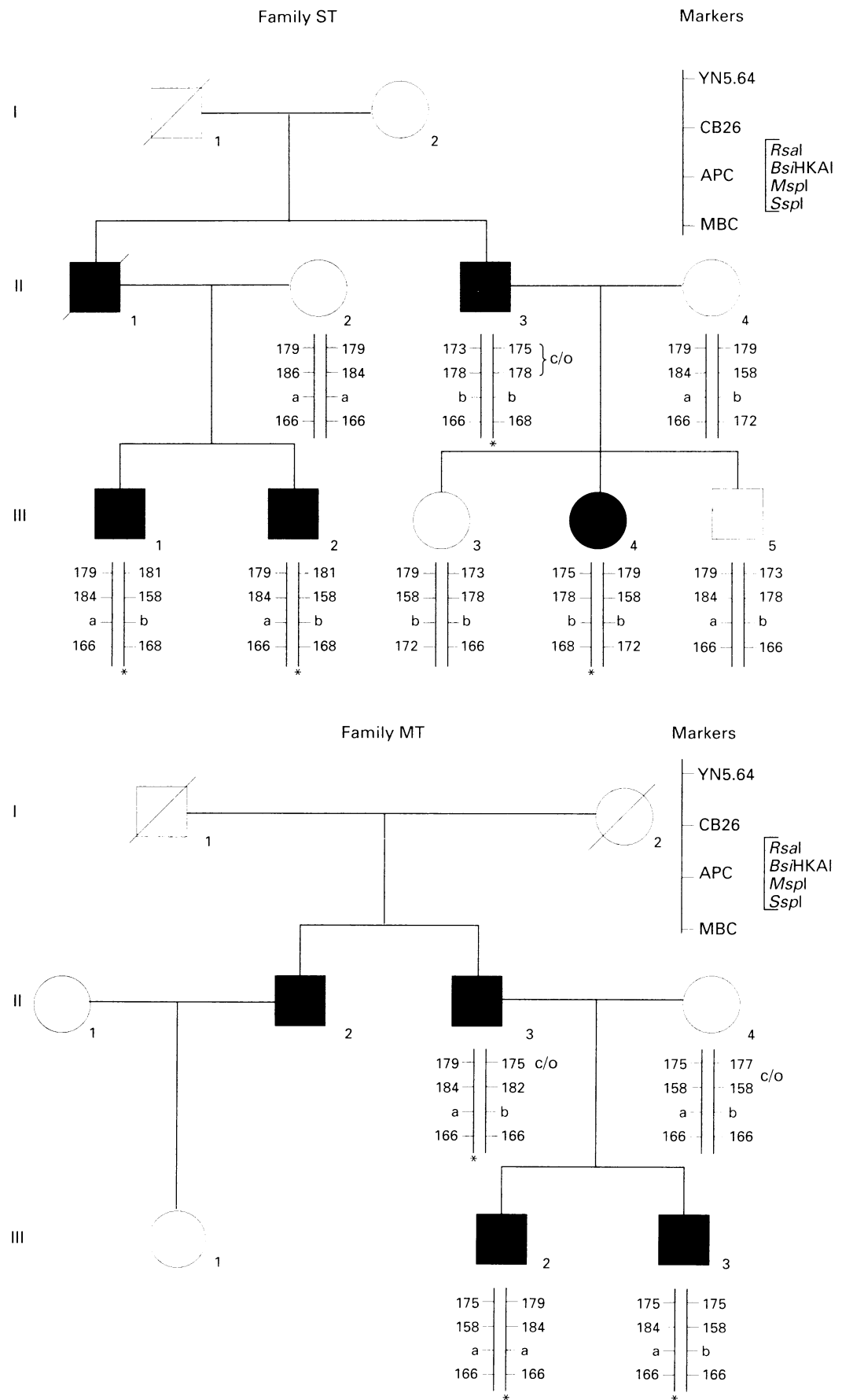
The PCR mixtures consisted of 100 ng of genomic DNA, 20 pmol of each primer, 0.2 mmol/l dNTPs, and 0.75 U AmpliTaq DNA polymerase (Perkin-Elmer) in 1 × reaction buffer in a volume of 20 µl, overlaid

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Pedigrees of families ST and MT. Shaded symbols indicate affected members with polyps and open symbols are unaffected or have status unknown at the time of study. The solid lines below each symbol indicate the two haplotypes obtained for each person. The order of the markers in haplotypes are from centromere to telomere. For each (CA)_n marker we show the size of the two alleles in bp. The disease associated haplotype is represented by an asterisk. The recombination sites are indicated by "c/o". For APC intragenic haplotypes a: ++-- and b: --++.

with mineral oil. PCR conditions are described elsewhere.^{15-17 23-25} To detect intragenic RFLPs, subsequent enzymatic digestions were performed according to the manufacturer's instructions with *Rsa*I,²³ *Bst*HKA1,²³ *Msp*I,²⁴ and *Ssp*I,²⁵ respectively and the resulting fragments were electrophoresed through a 2% agarose gel.

To amplify (CA)_n flanking markers we used fluorescent labelled primers, and for gel analysis 1 µl of fluorescent PCR products were combined with 0.4 µl of internal lane size standard (ROX 2500, ABI), and mixed with 4 µl of formamide with dextran blue. Samples were electrophoresed on a 6% polyacrylamide gel in a 373A DNA sequencer (ABI). The standard is used to create a calibration curve within each gel lane. The unknown molecular lengths of PCR products are automatically calculated from the calibration curve using GENESCAN 672 software.

In the figure, we present two pedigrees under study in our laboratory showing haplotypes constructed with results obtained by the above methods. The order of the (CA)_n markers in the haplotypes are: cen-YN5.64-CB26-APC-MBC-tel. Based on the individual information of each intragenic RFLP (*Rsa*I, *Bst*HKA1, *Msp*I, *Ssp*I), haplotypes were drawn as a: + + - - and b: - - + +, respectively.

The pedigree of families ST and MT show recombinant events leading to the localisation of the CB26 marker. The pedigree of family ST shows that the offspring III-3 and III-5 have not inherited the disease associated haplotype from their father, and are therefore at low risk, but their sister does carry it. We found two disease associated haplotypes for the markers YN5.64 and CB26. The observed recombinant event involves both YN5.64 and CB26 markers and therefore we were able to clarify that the latter marker is centromeric to APC.

In the pedigree of family MT both patients III-2 and III-3 have inherited the same affected chromosomal region from their father, II-3. A recombinant event occurred in both II-3 and II-4. In II-4 we could not precisely place the recombinant event because there was a homozygous result for marker CB26. However, in II-3 we observed that the recombinant event involved only marker YN5.64. Therefore, we were able to place the marker CB26 (D5S299) between D5S82 and the APC gene.

In the 11 families, the study was informative for 12% of subjects using only intragenic RFLPs and increased to 90% when we used the three (CA)_n flanking markers. In one family (not shown) we observed a loss of (CA) units for the marker CB26 from an unaffected mother to her son. From our experience the use of different closely linked and polymorphic micro-satellite markers is a highly reliable method for presymptomatic diagnosis in the FAP families under study.

Although the use of haplotype segregation analysis is the most widespread test for presymptomatic diagnosis, its informativeness is still limited in some cases because (1) key family members may be unavailable; we found in two of our FAP families that DNA extracted from

preserved tissues of dead relatives would be crucial to extend the informativeness value; (2) linkage analysis cannot be applied when a new mutation is suspected, which applies to approximately 24% of affected subjects; and (3) linkage markers may also not be informative. To overcome these disadvantages a recently described protein truncated test (PTT test)²⁶ may now be set up as a parallel direct screening procedure.

In conclusion, the methodology of fluorescence based semiautomated DNA analysis was established to perform presymptomatic diagnosis in Portuguese FAP families for indirect genotyping of FAP with several markers with high accuracy. A protocol for the co-electrophoresis of all markers has now been set up in our laboratory for an even more cost effective procedure; the method is less time consuming with the same high accuracy.

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