Presumptive diagnosis in families with adenomatous polyposis using highly polymorphic dinucleotide CA repeat markers flanking the APC gene

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Abstract
A panel of five multiallelic and highly informative dinucleotide CA repeat markers flanking the APC gene was used for presymptomatic diagnosis of familial adenomatous polyposis coli (FAP). Marker regions were amplified by PCR. DNA fragments were separated by electrophoresis in denaturing polyacrylamide gels and visualised by ethidium bromide staining. Two or more markers were found to be informative in all nine families tested, and all 23 persons at risk could be diagnosed as affected or unaffected by the disease gene, the probability being > 99.9% in 14 cases from six families in which flanking markers were informative.

We found no indication for locus heterogeneity of the disease in our sample. The polyposis phenotype and its extracolonic manifestations co-segregate with a distinct haplotype determined by the markers flanking the APC gene. In one family with no remaining living affected members, we could infer the high risk haplotype from genotyping of first degree relatives. The segregation of this haplotype is consistent with the occurrence of CHRPEs in the progeny. In a sporadic case we made use of the typical early extracolonic manifestations of the disease (osteomas, desmoids) to identify the high risk haplotype.

We conclude from our experience that indirect genotyping of FAP with this particular panel of closely linked and highly polymorphic microsatellite markers is a rapid, efficient, and highly reliable method for presymptomatic diagnosis of FAP.

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Familial adenomatous polyposis (FAP) is one of the most frequent (around 1:10000) dominantly inherited predispositions to colon cancer with age dependent penetrance. 1,3 Typically, affected persons develop multiple polyps in the colonic epithelium during the second or third decade of life. One or more of these adenomas inevitably progress to adenocarcinoma if left untreated. Frequent examination of at risk subjects by sigmoidoscopy beginning in the early teens is therefore recommended in order to detect early adenomas and carry out prophylactic colectomy.

A valuable early predictive marker of the disease is bilateral congenital hypertrophy of the retinal pigment epithelium (CHRPE) which occurs in about 70 to 80% of FAP kindreds. 4,5 In addition, there are some other frequent and variably expressed extraintestinal manifestations associated with FAP, such as desmoid tumours, osteomas of the mandible and skull, and epidermoid cysts, previously defined as Gardner syndrome. 6-11 The genetic locus responsible for FAP including Gardner syndrome has been mapped to chromosome 5q21-22. 11,12 Subsequently, the putative tumour suppressor gene called APC (adenomatous polyposis coli) was isolated by positional cloning 14-18 and shown to include a large coding region divided into 15 exons. The inactivating mutations transmitted through the germline of patients are distributed all over the 5’ half of the coding region and are unique in most FAP families. Exceptions are two 5 bp deletions in the 5’ region of exon 15 which, however, together occurred in no more than 15 to 20% of unrelated kindreds examined so far. 15,22 Thus, direct screening for the individual mutation is not yet practical for routine diagnosis. Until recently, indirect diagnosis of disease gene carriers was performed using APC gene linked RFLP markers which were, however, not sufficiently informative in many families. 21,22 Recently, several highly polymorphic and informative microsatellite markers flanking the APC gene were isolated, 19-23 two of which (MBC and LNS) have already been used for diagnostic testing in recent studies. 23,24 We have used five of these markers for presymptomatic diagnosis of at risk members from nine FAP families. At least two of these markers were informative in each of these families and diagnostic probabilities of over 99.9% could be obtained in most cases.

Methods

FAMILIES AND CLINICAL DIAGNOSIS

Members of 14 FAP families from the Black Forest and upper Rhine area were referred to our institute for genetic counselling during the last three years. Nine families who finally accepted DNA diagnosis were included in this study. Case histories and pedigree information were obtained by careful interviews. Altogether, the families comprised 69 persons including 23 persons at a 50% a priori risk.
All families except one had at least two persons in two generations in whom polyposis was diagnosed by clinical criteria. In one family (FAP-6) the index patient, a mother of three children, is an isolated case, presumably carrying a new mutation. In most families the affected members and at risk persons over 5 years of age were also examined by appropriate techniques for the presence of CHRPEs, or other early extracolonic manifestations of FAP, such as osteomas and desmoid tumours.

Several of the affected persons have already undergone prophylactic colectomy or have been operated on for colorectal cancer. During the last three generations, at least 12 members of these families died of the disease aged between 25 and 60 years. In one family (FAP-10) all affected subjects had died and were thus not available for DNA analysis.

DNA isolation, PCR amplification, and electrophoretic separation of allelic products of microsatellite markers
DNA was isolated from lymphocytes by 10 ml EDTA anticoagulated venous blood by proteinase K digestion, phenol-chloroform extraction, and ethanol precipitation according to a procedure modified from Madison et al.

Amplification of the (dc-dA) dinucleotide repeat markers (see characteristics summarised in table 1) was performed in 50 μl PCR assays containing 0.5 μg genomic DNA, 0.2-1 μmol/l of each primer, 0.2 μmol/l dNTPs, and 2.5 U Taq polymerase (Amersham) in Taq polymerase buffer (Amersham). Samples were processed through an initial denaturation step at 94°C for five minutes and 30 cycles under variable primer specific conditions, with denaturation at 94°C for one minute, annealing under primer specific conditions (JW25: 50°C, three minutes; MBC: 58°C, two minutes; LNS: 60°C, one minute; YN5.64:53°C, one minute; CB26: 55°C, 1.5 minutes), and extension at 72°C for one minute followed by a final elongation step at 72°C for seven minutes in a programmable thermoblock (Techne PHC1). LNS together with CB26 or MBC, and YN5.64 together with JW25 marker regions, were frequently amplified in duplex PCR assays. PCR products were precipitated with ethanol, dried, and dissolved in 6 μl of 0.5 X TBE (Tris-borate-EDTA) buffer. Then 4 μl of formamide/dye gel loading buffer were added, the samples denatured at 95°C for five minutes, and immediately snap cooled in an ice bath. Electrophoresis was performed through vertical 16 x 20 cm long and 1 mm thick 8 to 10% denaturing (8 mol/l urea) polyacrylamide gels in 1 X TBE at 500 V for three to five hours. Before application of the samples, the buffer of the upper reservoir was heated to 60°C. The temperature of the lower gel during the run at the voltage used was 50 to 55°C.

After electrophoresis, gels were stained with ethidium bromide (1 μg/ml in TBE buffer) for 10 minutes, and then rinsed under running water for another 10 minutes. Gels were photographed on a UV transilluminator (302 nm) with a Polaroid MP4 camera. The data were collected and haplotypes constructed by inspection.

Results and discussion
Blood samples were obtained during the last three years from 69 members of nine families including 20 subjects affected according to clinical criteria and 23 subjects who were at a 50% a priori risk.

Initially, indirect genotyping was performed by the earlier available APC gene flanking and intragenic RFLP markers, but these were temperature sensitive in only about 50% of cases (C Jung, unpublished data). When several highly polymorphic dinucleotide CA repeat markers around and tightly linked to the APC gene became available (for order and characteristics see table 1), we established a rapid and convenient non-radioactive method for separation and detection of PCR amplified marker allele fragments in denaturing medium sized polyacrylamide gels. An example of such a gel separation is shown in fig 1, and results obtained by this indirect gene diagnosis are summarised in table 2. Two or more markers were found to be informative in all families tested, and in six families these markers flank both sides of the APC gene. Thus, in 14 of 23 cases the diagnostic reliability is over 99.9% when estimated from recombination fractions and the low probability of double recombination between these closely linked flanking markers. Moreover, assuming interference within such short genetic distances (≤ 7 cm), the accuracy of diagnosis should approach 100%.

A remaining source of error when using indirect DNA diagnostic methods is genetic heterogeneity (locus heterogeneity) occasionally observed by others. We have found no evidence for locus heterogeneity in our sample. In all our cases the disease phenotype, including its extracolonic manifestations, cosegregates with these markers. Therefore, in such cases the disease may be inherited in a non-penetrant fashion.

### Table 1 Dinucleotide CA repeat markers flanking the APC gene used in this study

<table>
<thead>
<tr>
<th>Marker</th>
<th>Locus</th>
<th>No of alleles</th>
<th>PIC</th>
<th>Approximate physical or genetic distance from the APC gene</th>
</tr>
</thead>
<tbody>
<tr>
<td>CB26</td>
<td>DSS299</td>
<td>7</td>
<td>0.66</td>
<td>&gt; 5 cm proximal*</td>
</tr>
<tr>
<td>YN5.64</td>
<td>DSS82</td>
<td>6</td>
<td>0.07</td>
<td>4-5 cm proximal</td>
</tr>
<tr>
<td>LNS</td>
<td>DSS8246</td>
<td>13</td>
<td>0.83</td>
<td>50 kb distal†</td>
</tr>
<tr>
<td></td>
<td>DSS5103</td>
<td>4</td>
<td>0.49</td>
<td>500 kb distal</td>
</tr>
<tr>
<td>MBC</td>
<td>MCC gene</td>
<td>7</td>
<td>0.78</td>
<td>2 cm distal</td>
</tr>
</tbody>
</table>

* This marker has not been precisely localised yet.
† The question of whether LNS and MBC markers are located distal or proximal to the APC gene is still controversial. We have used the orientation recently determined by Ward et al. The use of this information was essential to the design of the above primers.
gated with a distinct haplotype determined by the markers flanking the APC gene (for example, figs 2 and 5). Some pedigrees representative of the diagnostic situations encountered in our sample are shown in figs 2 to 5.

Fig 2 shows the pedigree of a typical three generation FAP family (FAP-3) in which osteomas as an extracolonic manifestation of the disease were found in all affected subjects. The at risk child (E6) who inherited the marker haplotype associated with the disease gene from his affected father has already developed an osteoma of the mandible in contrast to the older at risk subjects, D3 and C8, who did not inherit the FAP predisposing haplotype from their mothers. (The child E7, a 4 year old boy with a high risk for FAP, has not yet been examined for osteomas by radiography).

The concordance between marker haplotype status and early development of extracolonic manifestations of FAP is important for risk assessment in the case of family FAP-6 (fig 3).

In this family, three children of an affected mother, who presumably carries a new mutation, inherited an identical maternal haplotype which could be associated with either a low or a high risk. Since each of the three children (5, 7, and 11 years old) already shows one of the typical early extraintestinal lesions, also

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**Table 2** Summary of results from indirect gene diagnosis of at risk subjects from FAP families using CA repeat markers

<table>
<thead>
<tr>
<th>Family</th>
<th>No of subjects tested</th>
<th>No of affected subjects</th>
<th>No of subjects at risk</th>
<th>No of at risk subjects determined to be</th>
<th>Markers informative for at risk subjects</th>
<th>Reliability of DNA diagnosis (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>FAP-1</td>
<td>11</td>
<td>3</td>
<td>1</td>
<td>2</td>
<td>A1: JW25, MBC, YN5.4L, YN5.64</td>
<td>99-998</td>
</tr>
<tr>
<td></td>
<td></td>
<td>4</td>
<td>2</td>
<td></td>
<td>E1-E3: JW25, YN5.64, CB26</td>
<td>99-91</td>
</tr>
<tr>
<td></td>
<td></td>
<td>4</td>
<td>2</td>
<td></td>
<td>B8: JW25, LNS, YN5.64</td>
<td>99-998</td>
</tr>
<tr>
<td></td>
<td></td>
<td>4</td>
<td>2</td>
<td></td>
<td>D3: JW25, MBC</td>
<td>99-8</td>
</tr>
<tr>
<td></td>
<td></td>
<td>4</td>
<td>2</td>
<td></td>
<td>C8: LNS, YN5.64</td>
<td>99-998</td>
</tr>
<tr>
<td></td>
<td></td>
<td>4</td>
<td>2</td>
<td></td>
<td>E5, E7: LNS, JW25, YN5.64</td>
<td>99-998</td>
</tr>
<tr>
<td>FAP-2</td>
<td>6</td>
<td>3</td>
<td>1</td>
<td>1</td>
<td>D3: JW25, MBC</td>
<td>99-8</td>
</tr>
<tr>
<td></td>
<td></td>
<td>1</td>
<td>1</td>
<td></td>
<td>B8: JW25, LNS, YN5.64</td>
<td>99-998</td>
</tr>
<tr>
<td></td>
<td></td>
<td>1</td>
<td>1</td>
<td></td>
<td>D3: JW25, MBC</td>
<td>99-8</td>
</tr>
<tr>
<td>FAP-3</td>
<td>11</td>
<td>3</td>
<td>1</td>
<td>1</td>
<td>D3: JW25, MBC</td>
<td>99-8</td>
</tr>
<tr>
<td></td>
<td></td>
<td>1</td>
<td>1</td>
<td></td>
<td>B8: JW25, LNS, YN5.64</td>
<td>99-998</td>
</tr>
<tr>
<td>FAP-4</td>
<td>6</td>
<td>2</td>
<td>2</td>
<td>1</td>
<td>D3: JW25, MBC</td>
<td>99-8</td>
</tr>
<tr>
<td></td>
<td></td>
<td>2</td>
<td>2</td>
<td></td>
<td>B8: JW25, LNS, YN5.64</td>
<td>99-998</td>
</tr>
<tr>
<td>FAP-6</td>
<td>5</td>
<td>1</td>
<td>1</td>
<td>0</td>
<td>D3: JW25, MBC</td>
<td>99-8</td>
</tr>
<tr>
<td></td>
<td></td>
<td>1</td>
<td>1</td>
<td></td>
<td>B8: JW25, LNS, YN5.64</td>
<td>99-998</td>
</tr>
<tr>
<td>FAP-7</td>
<td>4</td>
<td>1</td>
<td>1</td>
<td>0</td>
<td>D3: JW25, MBC</td>
<td>99-8</td>
</tr>
<tr>
<td></td>
<td></td>
<td>1</td>
<td>1</td>
<td></td>
<td>B8: JW25, LNS, YN5.64</td>
<td>99-998</td>
</tr>
<tr>
<td>FAP-10</td>
<td>11</td>
<td>2</td>
<td>2</td>
<td>1</td>
<td>D3: JW25, MBC</td>
<td>99-8</td>
</tr>
<tr>
<td></td>
<td></td>
<td>2</td>
<td>2</td>
<td></td>
<td>B8: JW25, LNS, YN5.64</td>
<td>99-998</td>
</tr>
<tr>
<td>FAP-12</td>
<td>9</td>
<td>3</td>
<td>3</td>
<td>1</td>
<td>D3: JW25, MBC</td>
<td>99-8</td>
</tr>
<tr>
<td></td>
<td></td>
<td>4</td>
<td>4</td>
<td></td>
<td>B8: JW25, LNS, YN5.64</td>
<td>99-998</td>
</tr>
<tr>
<td>Total</td>
<td>69</td>
<td>20</td>
<td>10</td>
<td>23</td>
<td>K5-K7: LNS, JW25</td>
<td>99-95</td>
</tr>
</tbody>
</table>

* At risk subjects are indicated by their code numbers (see, for example, figs 2-5).
† Estimated from the recombination fractions.
‡ See text and fig 3.
Presymptomatic diagnosis in families with adenomatous polyposis using highly polymorphic dinucleotide CA repeat markers flanking the APC gene

Figure 3 Pedigree, clinical status, and CA repeat marker haplotype segregation in family FAP-6. In this family the affected subject F2 is an isolated case and thus probably carries a new mutation in the APC gene. Her three children (G1-G3) inherited an identical haplotype from her (and also from their father, F8). Since all three children already show typical extracolonic manifestations of FAP, either osteomas (OS+) or desmoids (DM+), it is most likely that their maternal haplotype is the affected one (tentatively indicated by the blackened gene symbol). For other explanations of symbols and graphic details see legend to fig 2.

Figure 4 Pedigree and CA repeat marker haplotype segregation in family FAP-10. In this family all affected subjects were dead. The haplotype of the male in the second generation who died of colon cancer at the age of 26 has been reconstructed and is given in brackets. The at risk girl (H2), in contrast to her brother (H3) and sister (H4), inherited from her father a grandmaternal haplotype (see H1) which is also present in her unaffected uncle (G8). CH refers to CHRPE (+, present; -, absent; ?, not yet examined). For explanation of other symbols and graphic details see fig 2.

Figure 5 Combined pedigrees and CA repeat marker haplotype segregation in related families FAP-1 and FAP-11. The two pedigrees are connected through the dead affected great grandmother (FAP-1, left) and grandmother (FAP-11, right) who were sisters. An identical marker haplotype associated with the disease gene is present in all affected members of both families (A7, A4, A2 in FAP-1 and H7, J1 in FAP-11). In the fourth generation of FAP-1 the at risk subjects E2 and E3 inherited the high risk haplotype. Recomb, recombination event between markers MBC and JW25.
expressed in their mother, they have to be regarded as being at high risk. It is very unlikely in this family that the “Gardner” phenotype is not the result of an APC gene mutation, since all three children of the husband inherited the same maternal APC gene flanking marker haplotype and all are similarly affected by the typical FAP associated extracolonic lesions. Search for the causative APC gene mutation in this family is under way to confirm this assumption.

The pedigree in fig 4 represents the case of a family (FAP-10) in which all affected members had not died already of the disease and thus were not available for DNA typing. Therefore, the genotypic of the affected dead father had to be inferred from his relatives, and the segregation pattern allowed for a unique haplotype assignment. The daughter (H2) obviously inherited from her father a grandmaternal haplotype which is also present in her unaffected uncle (G8). In contrast, her brother (H3) and sister (H4) inherited from their father the grandpaternal haplotype which is not present in the unaffected uncle and, therefore, must be the high risk haplotype. This is in agreement with the ophthalmological finding of bilateral CHRPEs in H3, but not in H2 (the younger sister H4 has not been examined for CHRPE yet).

An interesting case is represented by two relatively large families (FAP-1 and FAP-11) which were initially genotyped separately, but later incidentally turned out to be related through their dead affected great grandmother (FAP-1) and grandmother (FAP-11), respectively. The marker alleles of the members of both families were subsequently compared by electrophoresis of each PCR sample in the same gel. This comparative analysis showed an identical marker haplotype associated with the disease gene in all affected subjects from both families. In addition, the two at risk persons (E2 and E3) in the fourth generation of FAP-1 carry this same haplotype. Thus, the inheritance of the disease gene can be traced unambiguously through four generations.

We can conclude from our experience that indirect gene diagnosis of at risk subjects in FAP families with this panel of closely linked, highly polymorphic, and informative microsatellite markers flanking the APC gene is a rapid, efficient, and highly valuable method for presymptomatic diagnosis of FAP.

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