The genotype of a new linked DNA marker, MP6d–9, is related to the clinical course of cystic fibrosis

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Abstract
The clinical symptoms of a cohort of cystic fibrosis patients were related to their genotypes using RFLPs shown with MspI and the closely linked DNA marker MP6d–9. In the majority of CF chromosomes, the restriction site for MspI was present, and the genotype 2/2 was found most often in patients who were severely affected by the disease. The genotype 1/2 was significantly over-represented in patients with very mild clinical manifestations, including pancreatic sufficiency, absence of meconium ileus, and absence of Pseudomonas colonisation. When pancreatic dysfunction was present, the 1/2 genotype was associated with a mild form, while the 2/2 genotype was found in patients with severe insufficiency. None of our patients had the 1/1 genotype. These results indicate that the newly isolated MP6d–9 marker correlates with some important symptoms of cystic fibrosis.

Cystic fibrosis (CF) is an autosomal recessive disease with a prevalence of about 1 in 2000 live births and a carrier frequency of approximately 1 in 23 in Caucasian populations.

Linkage analysis has localised the CF mutation close to a protein variant, and then assigned it to the long arm of chromosome 7 using DNA markers. Several DNA probes tightly linked to the CF gene are currently in use for carrier detection and prenatal diagnosis in families with at least one affected child.

The newly isolated marker, pMP6d–9 (D7S399), maps between the DNA sequences recognised by pKM19 (D7S23) and pJ3.11 (D7S8). pMP6d–9 is the closest, to date, to CF and is the one that shows the highest degree of non-random allelic association (linkage disequilibrium) in the Italian population. We report here a study on the correlation between the allelic systems determined by pMP6d–9, pXV2C, pKM.19, and pJ3.11 and the clinical manifestations of cystic fibrosis, including meconium ileus, pancreatic function, Pseudomonas colonisation, lung involvement, congestive cardiomyopathy, diabetes, biliary cirrhosis, and nasal polyposis.

Subjects and methods
PATIENTS
Data were collected from 86 unrelated Italian patients attending the CF centres of Verona and Rome. Blood samples were obtained in each family from parents and one affected child for DNA genotyping. CF diagnosis was confirmed with at least two positive sweat tests, performed according the method of Gibson and Cooke.

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Received for publication 8 July 1989.
Revised version accepted for publication 14 August 1989.
DNA GENTYPING AND STATISTICAL ANALYSIS
Genomic DNA was prepared from whole blood by standard methods. Restriction endonuclease digestion, transfer of DNA fragments to nylon or nitrocellulose membranes (Hybond–N and Hybond–CE, Amersham International), hybridisation, and autoradiography were carried out as previously described.13 The pMP6d–9/\textit{MspI},10 pKM19/PstI,9 pXV2C/TaqI,7 and pJ3.11/\textit{MspI} RFLPs were determined for each family. Different tests (normal test, $\chi^2$ test, and Fisher's exact test) were used to estimate the difference of proportion. The linkage disequilibrium analysis has been reported elsewhere.11

CLINICAL ASSESSMENT
Clinical data were obtained from patients basically according to a protocol previously reported,13a in which a form, designed by several of the cooperating clinical groups, was used to collect information for each patient. Ileal obstruction at birth requiring medical or surgical therapy was defined as meconium ileus. Pancreatic insufficiency was defined on the basis of clinical course, steatorrhea (stool fat g/day), or fat balance (coefficient of fat absorption), or steatorcit, or all of these.14 The pulmonary function was established by analysing several successive chest x-rays according to Chrispin and Norman.15 The slope and a Y intercept of the regression line of the radiological score were used for the analysis, dividing the patients into three classes (mild, moderate, and severe lung involvement). \textit{Pseudomonas} colonisation (established through serial sputum cultures) was defined according to age at first appearance. The presence of other complications, such as diabetes, biliary cirrhosis, or nasal polyposis, was also registered. The mean age of our patient sample was 9 years 7 months.

Results
Table 1 shows the MP6d–9 genotypes of a sample of our CF patients: 63% had the 2/2 genotype and 37% the 1/2 genotype. The 1/1 genotype was not seen in this group of 86 patients, but it was present in 7% of their carrier parents. This difference is statistically significant. Linkage disequilibrium analysis for this population has been reported elsewhere.11

Table 2 records the distribution of the clinical manifestations in our patients. Lung involvement was present in all patients and in the majority pancreatic insufficiency was present and meconium ileus was absent. No significant association of genotypes with the severity of pulmonary involvement, or with meconium ileus, \textit{Pseudomonas} colonisation, nasal polyposis, or other complications was observed.

The analysis of the relation between MP6d–9 genotypes and pancreatic dysfunction is shown in table 3. This table shows that there is a significant difference in the distribution of the MP6d–9 genotypes, with an overrepresentation of the 1/2 genotype in pancreatic sufficient, and of the 2/2 genotype in pancreatic insufficient patients. The mean ages of the patients included in the two groups were 8–7 years (insufficiency) and 14–6 years (sufficiency). All cases with pancreatic sufficient had therefore reached an age at which the total or partial conservation of function allowed this group to be differentiated from the other. A similar analysis was performed with the pXV2C/TaqI, pKM19/PstI, and pJ3.11/\textit{MspI} RFLPs. A significant association of the KM19 genotype 2/2 with pancreatic insufficient was found, but no significant relation with the other two polymorphisms. It appears that haplotype 2,2 for J3.11 and haplotype 1,1 for pXV2C are also related to pancreatic dysfunction. It should be noted that the number of observations for the J3.11 polymorphism was the lowest.

Table 1 MP6d–9 genotypes of CF patients.

<table>
<thead>
<tr>
<th>Genotype</th>
<th>Patients</th>
<th></th>
<th></th>
<th>Parents</th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>No</td>
<td>%</td>
<td>No</td>
<td>%</td>
<td>No</td>
<td>%</td>
</tr>
<tr>
<td>1,1</td>
<td>0*</td>
<td>0</td>
<td>10*</td>
<td>7</td>
<td></td>
<td></td>
</tr>
<tr>
<td>1,2</td>
<td>32</td>
<td>37</td>
<td>103</td>
<td>70</td>
<td></td>
<td></td>
</tr>
<tr>
<td>2,2</td>
<td>54</td>
<td>63</td>
<td>34</td>
<td>23</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total</td>
<td>86</td>
<td>100</td>
<td>147</td>
<td>100</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Some parental samples were not available for analysis. * = p<0.05.

Table 2 Distribution of the clinical manifestations of CF patients.

| Clinical manifestation | Present | | | Absent | | | | Uncertain | | | |
|------------------------|---------|-----|-----|---------|-----|-----|---------|-----|-----|---------|-----|-----|
|                        | No      | %   | No  | %   | No  | %   | No  | %   | No  | %   |
| Lung involvement       | 54      | 100 | 0   | 0   | 0   | 0   |      |     |      |     |
| Meconium ileus         | 16      | 19  | 63  | 73  | 7   | 8   |      |     |      |     |
| Pancreatic insufficiency | 69   | 80  | 11  | 13  | 6   | 7   |      |     |      |     |
| \textit{Pseudomonas} colonisation | 36 | 42  | 44  | 51  | 6   | 7   |      |     |      |     |
| Nasal polyposis        | 7       | 8   | 74  | 86  | 5   | 6   |      |     |      |     |
| Other complications    | 3       | 3   | 78  | 91  | 5   | 6   |      |     |      |     |
| (diabetes or cirrhosis) |        |     |      |     |      |     |      |     |      |     |

Percentage is computed by row.
The genotype of a new linked DNA marker, MP6d-9, is related to the clinical course of cystic fibrosis

Table 3 Distribution of genotypes of CF patients by pancreatic involvement.

<table>
<thead>
<tr>
<th>RFLP</th>
<th>Genotype</th>
<th>Sufficiency</th>
<th>Insufficiency</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>No</td>
<td>%</td>
</tr>
<tr>
<td>MP6d-9/MspI</td>
<td>1,2</td>
<td>7</td>
<td>26</td>
</tr>
<tr>
<td></td>
<td>2,2</td>
<td>4</td>
<td>7.5</td>
</tr>
<tr>
<td>KM19/PstI</td>
<td>1,1</td>
<td>2</td>
<td>/</td>
</tr>
<tr>
<td></td>
<td>2,2</td>
<td>7</td>
<td>27</td>
</tr>
<tr>
<td>XV2C/TaqI</td>
<td>1,1</td>
<td>4</td>
<td>9</td>
</tr>
<tr>
<td></td>
<td>1,2</td>
<td>8</td>
<td>23</td>
</tr>
<tr>
<td></td>
<td>2,2</td>
<td>1</td>
<td>/</td>
</tr>
<tr>
<td>J3.11/MspI</td>
<td>1,1</td>
<td>5</td>
<td>26</td>
</tr>
<tr>
<td></td>
<td>2,2</td>
<td>0</td>
<td>/</td>
</tr>
</tbody>
</table>

*p<0.03. /=total number of observations too low to compute percentage.

Table 4 Distribution of MP6d-9 genotypes of CF patients by severe or minimal pancreatic insufficiency.

<table>
<thead>
<tr>
<th>Genotype</th>
<th>Steatorrhoea</th>
<th>Fat absorption coefficient</th>
<th>Steatorrhoea</th>
<th>Fat absorption coefficient</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Light (&lt;2-6%)</td>
<td>Severe (&gt;6%)</td>
<td>Light (&lt;70%)</td>
<td>Severe (&lt;70%)</td>
</tr>
<tr>
<td>1,2</td>
<td>3*</td>
<td>11*</td>
<td>5</td>
<td>18</td>
</tr>
<tr>
<td>2,2</td>
<td>0</td>
<td>11*</td>
<td>6</td>
<td>17</td>
</tr>
</tbody>
</table>

*Fisher's exact test p=0.00275.

A further, more detailed analysis of the relation between the MP6d-9 genotype and the severity of pancreatic insufficiency is shown in table 4. When pancreatic insufficient patients were divided into two groups, according to the severity of the pancreatic dysfunction, it was found that the MP6d-9 1/2 genotype was overrepresented in the mild form, defined by <9 g/day steatorrhoea (without pancreatic enzyme supplementation), or by a steatorrhoea of <6%, or by a coefficient of fat absorption of >70%. This relation is highly significant for steatorrhoea, where the sample size is lowest, and almost significant (p=0.087) for steatorrhoea. Steatorrhoea has low specificity and high sensitivity and might depend, as well as steatorrhoea, on different dietary fat intake. Fat absorption, however, is independent of age, normalised for body weight, and calculated relative to known fat intake.

Table 5 shows the relation between MP6d-9 genotypes and a division of CF into severe and mild clinical forms. Pancreatic insufficiency, meconium ileus, and *Pseudomonas* colonisation were used as the major criteria to identify the two classes, the first including patients without any of the above manifestations, and the second including patients with one or more of them. The ages of the patients included in the two groups were 9-3 years (with) and 11-8 years (without). The lack of *Pseudomonas* colonisation, therefore, was not related to the age of the patients, as it would be a progressive change over time. A highly significant difference of distribution of MP6d-9 genotypes was found: genotype 1/2 is overrepresented in the first class, corresponding to minimal disease. This genotype was not seen in CF patients manifesting all three clinical features. The same analysis did not indicate significant correlations with genotype for any of the other RFLPs investigated. The MP6d-9 RFLP, therefore, showed a better ability to discriminate between mild and severe CF than other linked RFLPs. In addition, complications (diabetes and biliary cirrhosis) were observed only in patients with the MP6d-9 genotype 2/2.

**Discussion**

pMP6d-9 is the DNA marker most tightly linked to CF available at present. Therefore, this marker currently offers the best possibility of relating individual genotype to clinical presentation and course of cystic fibrosis. In Italian families, the correlation coefficient for linkage disequilibrium between MP6d-9 and KM19 is 0.73 and between MP6d-9 and XV2C is 0.43 on CF chromosomes. We have found that MP6d-9 allele 1 is associated with milder disease. Three of the most common features of cystic fibrosis were used as indicators of the degree of severity: pancreatic insufficiency, *Pseudomonas* colonisation, and meconium ileus. This last symptom is the earliest clinical manifestation of cystic fibrosis, and may be related to the decrease in pancreatic enzymes, to the decrease in water, to the increase in protein, or to the altered secretion of small intestinal mucus. The determination of the genotype of the patient or fetus will therefore indicate some of the most important symptoms of the disease. Genotype 2/2 correlates with
a more severe clinical spectrum and genotype 1/2 with a milder clinical picture. In an early attempt to correlate different clinical presentations of CF with genotype, Mornet et al. reported that the CF chromosomes have different J3.11 haplotypes in cases with and without meconium ileus. It was suggested that multiallelism was associated with the two forms of the disease. J3.11 is located at approximately 0.028 recombination units from CF. We found little, if any, association between MP6d-9 and J3.11. No relation between the occurrence of meconium ileus and alleles of the two closer markers, pXVC2 and pKM19, was found in this study, nor in another recent report, which did not confirm the J3.11/ileus association.

Unfortunately, no association of genotype with pulmonary function was found. A collaborative study in Italy has recently shown that specific pKM19 alleles associate preferentially in patients with and without pancreatic insufficiency. A similar conclusion has been reached independently for the W3D1.4/HindIII RFLP. W3D1.4 recognises the same region as XV2C. Its linkage disequilibrium with CF in our sample is 0.17 (unpublished data), compared with 0.30 for XV2C. Our sample showed the same preference of association of W3D1.4 with pancreatic disease as reported by the above group. The results reported here, relative to the new DNA marker MP6d-9, more closely linked to the CF mutation, confirm and extend these data, presenting a further and stronger relation between the MP6d-9 genotypes and severe and mild pancreatic insufficiency, as well as three different clinical variables, in each case haplotype 1/2 being preferentially associated with the mild form. Three families with affected sibs were used in this study. Variation in severity (but concordance in pancreatic function) was observed in all of them.

In previous studies of CF families from southern Europe we have shown the presence of at least one further ‘CF haplotype’ in addition to the one commonly found in over 85% of northern European chromosomes, indicating the occurrence of a second CF mutation. The observations here support this conclusion and could tentatively be explained by the presence of two different mutations in a single ‘cystic fibrosis gene’: a ‘mild’ one associated with the MP6d-9 allele 1 and a ‘severe’ one associated with the MP6d-9 allele 2. These data would be most compatible with mutations in a coding sequence that result in two different amino acid substitutions in a polypeptide chain with different phenotypic effects, as is found in the haemoglobinopathies. Patients with the 2/2 genotype would be affected more seriously than those with the 1/2 genotype, as we observed. We hypothesise that a subject with the 1/1 genotype would not present with classical CF at all, but perhaps with a less severe disease.

We wish to thank Professor G Mastella for encouragement and helpful collaboration; M T Miscio, R Galavotti, P Lorenzi, and M Arena for expert technical assistance; Drs J Buchanan, M Buchwald, and L C Tsui for generously making data available in advance of publication; the Cystic Fibrosis Centre of Verona, Italy; the Italian Ministry of Public Education; the Golden Products (Italy); the Spanish Ministry of Social Security (89/0563); the Spanish Ministry of Education and Science (PB87–0074); and the Cystic Fibrosis Research Trust (UK) for support. PG and AS were recipients of fellowships from the Cystic Fibrosis Centre of Verona, AR from the Italian Society of Prenatal Medicine.

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doi: 10.1136/jmg.27.1.17

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