Familial screening for genetic haemochromatosis by means of DNA markers

J Yaouanq, A El Kahloun, M Chorney, A M Jouanolle, V Mauvieux, M Perichon, M Blayau, P Pontarotti, J Y Le Gall, V David

Abstract
Genetic haemochromatosis (HFE) is a frequent and potentially fatal disease. Early phlebotomies may prevent complications. The recessive gene for HFE is unknown but closely linked to the HLA-A locus. No direct test for homozygosity for HFE is currently available, apart from HLA typing within the family of a patient with confirmed HFE.

During a reverse genetic approach to identify the gene, we found three anonymous genomic probes (P3, P5, and I.82) derived from previously cloned YACs and physically mapped in the HLA class I region. P3 and P5 probes recognise 3 loci (P3A, P3B, P5) and I.82 one locus about 100 kb from HLA-A. Using five biallelic polymorphisms (I.82/BglII, P3B/EcoRV, P5/BstI, P5/HindIII, P3A/PstI), we tested 198 HLA typed subjects from the families of 22 haemochromatosis patients. The information from the five polymorphisms was sufficient to identify unequivocally extended haplotypes in all families. The restriction haplotypes cosegregate with the HFE allele and enable identification of genotypically identical sibs in all families studied.

The linked DNA markers described in this article avoid the disadvantages of HLA serological typing and can be used in genetic counselling of HFE families.

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Abstract
Genetic haemochromatosis (HFE) is an inherited disorder of iron metabolism characterised by excessive iron absorption and progressive iron loading of parenchymal organs. It is now known to be one of the most common recessive metabolic disorders in Caucasian populations, the prevalence of the gene being as high as 0-05 to 0-08 in some areas such as Brittany.1,2 The underlying biochemical defect of the disease is unknown, but the gene is located on the short arm of chromosome 6, closely linked to the HLA class I region and closer to the HLA-A than to the HLA-B locus.3,5

There is strong evidence that visceral complications may be prevented if phlebotomy is undertaken before tissue damage occurs. Patients treated in the early stage of the disease have a normal life expectancy, in contrast to cirrhotic and diabetic patients who die from complications including hepatocellular carcinoma.6 In the absence of a specific test for homozygosity for HFE, two strategies are used for early identification of HFE patients. Detection in asymptomatic patients in the population at large is based on raised transferrin saturation and serum ferritin levels. The upper limits for normal values are not clearly established, however, and these tests are not sensitive in young subjects and in females.7,8 Family screening is an effective means of identifying homozygotes before the disorder becomes manifest, since the proband's HLA haplotypes are used as markers within his family.9

Since 1988, we have been developing a reverse genetic approach in order to locate the haemochromatosis gene with precision and, if possible, define its characteristics. During the search for genetic markers around the gene, we identified three anonymous probes which physically map within the HLA class I region. These probes detect five biallelic restriction fragment length polymorphisms (RFLPs) which allow the definition of extended restriction haplotypes through family studies. Until the mutation(s) that cause haemochromatosis has been identified, we suggest the use of these restriction haplotypes as markers in family screening for genetic susceptibility to the disease.

Subjects, material, and methods
DNA analysis
Genomic DNA was isolated from peripheral white blood cells. Agarose gel electrophoresis, Southern blotting onto nylon membranes, and hybridisation conditions have been previously described.10 The following restriction enzymes were used: EcoRV, HindIII, PstI, and BglII.

Probes
Three probes detecting five biallelic RFLPs were used. The P3 and P5 probes have already been described.11 P3 recognises two loci, P3A and P3B. P3A shows a restriction polymorphism with PstI (alleles 1: 3-8 kb, allele 2: 3-5 kb). P3B displays two biallelic polymorphisms with EcoRV (alleles 2 and 5 of 16 and 6-8 kb, respectively), and PstI (alleles 3: 1-8 kb and allele 4: 1-5 kb). The P5 probe hybridises with a single locus displaying a polymorphism with HindIII (alleles 1 and 2 of 17 and 14-5 kb, respectively). P3A lies on the centromeric side of HLA-B; P3B and P5 are located 600 kb from HLA-A and 700 kb from HLA-B (fig 1). We subcloned the I.82 probe from the yeast artificial chromosome (YAC) B30, which contains the HLA-A locus. This YAC was a gift of D Chaplin (Washington University School of
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<table>
<thead>
<tr>
<th>Probe</th>
<th>Enzyme</th>
<th>Fragment (kb)</th>
<th>Controls</th>
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<tr>
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<td>BglII</td>
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<tr>
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<td>EcoRV</td>
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<td>P5</td>
<td>HindIII</td>
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<td>0.37</td>
</tr>
<tr>
<td>P3 (A)</td>
<td>PstI</td>
<td>14.5</td>
<td>0.548</td>
<td>0.33</td>
</tr>
</tbody>
</table>

Allele frequencies of the five probe/enzyme systems in the control population. PIC (polymorphism information content) = 1 – (p² + q² + 2pq²) where p and q are the frequencies of each allele in the controls.

Results and discussion

We have already reported the results of population studies which showed that linkage disequilibrium between the HFE gene and RFLPs at P3A, P3B, and P5 either does not exist or is weak, whereas there is a strong positive association between HLA-A3 and the P3B markers. We also found that the 7-4 kb allele (allele 2) detected by 1.82 is significantly more frequent in the haemochromatosis patients than in the controls (0.76 vs 0.53, p < 10⁻⁴), which suggests positive linkage disequilibrium between this allele and the haemochromatosis gene.

The segregation of the five polymorphisms (1.82/BglII, P3B/EcoRV, P3B/PstI, P5/HindIII, and P3A/PstI) was studied in 198 members of 22 haemochromatosis families which included 57 affected patients and 95 nuclear families. Given the high heterozygosity of each marker in the controls (table), it was likely that at least one of the subset of five polymorphisms would prove informative in most families. In fact, the restriction haplotypes were unequivocally reconstituted in all cases. In the pedigrees studied, there was no evident recombination between the HFE allele and any of the five molecular markers of the HLA class I region, nor between the HFE gene and HLA-A or B loci. The restriction haplotypes clearly cosegregate with the haemochromatosis allele without any discordance for disease in sibs with the same genotype. As shown in fig 2, once a homozygous form of genetic haemochromatosis has been confirmed in a proband (II.3), a genetic status can be assigned to his sibs: sibs who share two haplotypes with the proband can be identified as homozygotes (II.5), those who share only one haplotype are obligate heterozygotes (II.2), and those who have neither are non-carriers (II.1).

Until now, the family screening programme has consisted of both clinical examination to detect affected patients and HLA serotyping to define the genotypic HFE status of each of the proband’s relatives. However, the serological method of HLA typing poses certain problems, including the fact that few laboratories are experienced and the number of serotypes feasible per week in any one laboratory is limited because blood samples must be tested immediately and two working days are required for the test. Furthermore, accurate determination of HLA phenotypes may sometimes prove difficult: blank typing at a given locus may correspond either to homozgyosity for the sole antigen detected or to an undetectable antigen for which no antiserum is available; antisera are frequently multispecific and cross react with several antigenic determinants within the same locus. Such late and ambiguous immunological results may lead to HLA mistyping and modify the genotype analysis in families.

Theoretically, HLA class I probes or HLA-A, B, C locus specific probes should overcome such difficulties. However, patterns obtained with class I probes are so complex that identification of allelic series and allele segregation studies in families are in fact extremely difficult and the information yielded with locus specific probes is limited. Consequently, none of these probes is routinely used to refine HLA typing and none can be applied to genetic counselling of patients with HLA linked diseases.

SUBJECTS AND FAMILIES

Haemochromatosis patients were selected as follows: there was no secondary cause of iron overload, and 5 g or more of iron were removed by quantitative weekly phlebotomies in males and 2 g or more in females.

The families, who had been previously HLA typed for HLA-A/B antigens, were screened with DNA probes. No recombined HLA-A and B haplotypes were observed. In addition, concordance for disease was confirmed in all the HLA identical sibs of the probands in the 10 pedigrees with more than one affected patient. The restriction haplotypes were deduced by hand using the HFE probe in each family to establish the linkage phase.

The frequencies of the two alleles detected by each probe were calculated after direct counting in 61 to 82 random, unrelated, normal controls taken from the population in Brittany. The polymorphic information content (PIC) for each polymorphism was based on the formula: 

\[
PIC = 1 - (p^2 + q^2 + 2pq^2)
\]

where p and q are the frequencies of each allele in the controls.

Figure 1 Schematic representation of the HLA class I region and physical location of loci P3A, P3B, P5, and 1.82.
step will be identification of the gene. If a single or very few mutations are responsible for the disorder, the use of direct genetic probes will be feasible. If, however, there are several mutations, linkage testing will probably remain an important tool in genetic counselling, as in many other inherited metabolic disorders.

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