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, P3B/PstI, 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 restriction 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.

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.^{1,7,8} 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.^{3,9}

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* (allele 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* (allele 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

Unité INSERM 155,
Paris, France.
J Yaouanq

Laboratoire de
Biochimie Médicale
B, CHU, Rennes,
France.

A El Kahloun
A M Jouanolle
V Mauvieux
M Perichon
M Blayau
J Y Le Gall
V David

Pennsylvania State
University, Hershey,
PA 17033, USA.
M Chorney

Centre Immunologie
INSERM/CNRS,
Marseille, France.
P Pontarotti

Correspondence to
Dr David, Laboratoire de
Biochimie Médicale B,
Faculté de Médecine,
2 Avenue du Pr Léon
Bernard, 35043 Rennes
Cedex, France.

Received 26 July 1991.
Revised version accepted
11 October 1991.

Figure 1 Schematic representation of the HLA class I region and physical location of loci P3A, P3B, P5, and I.82.

Medicine, St Louis, MO). We then located its position in genomic DNA by pulse field electrophoresis. I.82 is a 4.4 kb *Pst*I fragment at about 100 kb from *HLA-A*. It displays a restriction site polymorphism with *Bgl*II (allele 1: 15 kb and allele 2: 7.4 kb).

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 proband 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: $PIC = 1 - (p^2 + q^2 + 2p^2q^2)$ (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 I.82 is significantly more frequent in the haemochromatosis patients than in the controls (0.76 v 0.53, $p < 10^{-3}$), which suggests positive linkage disequilibrium between this allele and the haemochromatosis gene.¹²

The segregation of the five polymorphisms (I.82/*Bgl*II, P3B/*Eco*RV, P3B/*Pst*I, P5/*Hind*III, and P3A/*Pst*I) was studied in 198 members of 22 haemochromatosis families which included 37 affected patients and 50 nuclear families. Given the high heterozygosity of each marker in the controls (table), it was likely that at least one from 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 serotypings 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 homozygosity 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.¹³

Theoretically, HLA class I probes or HLA-A, B, C locus specific probes should overcome such difficulties.¹⁴⁻¹⁶ However, patterns obtained with class I probes are so complex^{17,18} 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.

Allele frequencies of the five probe/enzyme systems in the control population. PIC (polymorphism information content) = $1 - (p^2 + q^2 + 2p^2q^2)$ where p and q are the frequencies of the two alleles.

Probe	Enzyme	Fragment (kb)	Controls	PIC
I.82	<i>Bgl</i> II	15	0.467	0.37
		7.4	0.533	
P3 (B)	<i>Eco</i> RV	16	0.585	0.36
		6.8	0.414	
P3 (B)	<i>Pst</i> I	1.8	0.788	0.28
		1.5	0.211	
P5	<i>Hind</i> III	17	0.451	0.37
		14.5	0.548	
P3 (A)	<i>Pst</i> I	3.8	0.692	0.33
		3.5	0.307	
		3.5	0.307

Figure 2 (A) Example of a family study. The arrow indicates the proband. Haplotypes are constituted by the following polymorphisms (from top to bottom): I.82/BglII, P3B/EcoRV, P3B/PstI, P5/HindIII, and P3A/PstI. Haplotypes for subject II.1 were deduced from the genotypes of the offspring. The patterns observed are given in (B) (I.82/BglII), (C) (P3B/EcoRV), (D) (P3B/PstI alleles 3-4 and P3A/PstI alleles 1-2), and (E) (P5/HindIII).

Familial screening for disease by means of linked DNA markers that are easy to interpret (biallelic RFLPs, variable nucleotide tandem repeats, dinucleotide repeats) is now a strategy used in many mendelian disorders. Two biallelic markers may be sufficient provided that both markers have one allele in strong linkage disequilibrium with the gene, as has been shown for cystic fibrosis.¹⁹ At the moment, only one marker (I.82 allele 2) is in linkage disequilibrium with the HFE gene and the other four polymorphisms serve to increase informativeness. As more probes near the HFE gene become available, strategies can be developed to use only those probes that yield the greatest amount of information. The last

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.

This work has supported in part by grants from the Institut National de la Santé et de la Recherche Médicale (CRE No 91 1003), from the Ministère de la Recherche et de la Technologie (89/0751 to P Pontarotti and 90.C.0404 to V David), from the Association Française contre les Myopathies (AFM), and from the Fondation Langlois (Rennes). A El Kahloun is supported by AFM. We thank Professors H Allannic, P Brissot, Y Deugnier, and Dr O Nouel for providing patients. We thank also I Gicquel and M C Hazard for their technical assistance.

- Edwards CQ, Griffen LM, Goldgar D, Drummond C, Skolnick MH, Kushner JP. Prevalence of hemochromatosis among 11,065 presumably healthy blood donors. *N Engl J Med* 1988;318:1355-62.
- Simon M, Brissot P. The genetics of haemochromatosis. *J Hepatol* 1988;6:116-24.
- Simon M, Bourel M, Genetet B, Fauchet R. Idiopathic hemochromatosis. Demonstration of recessive inheritance and early detection by family HLA typing. *N Engl J Med* 1977;297:1017-21.
- Lalouel JM, Le Mignon L, Simon M, et al. Genetic analysis of idiopathic hemochromatosis using both qualitative (disease status) and quantitative (serum iron) information. *Am J Hum Genet* 1985;37:700-18.
- Simon M, Le Mignon L, Fauchet R, et al. A study of 609 haplotypes marking for the haemochromatosis gene: mapping the gene near the HLA-A locus and characters required to define a heterozygous population; hypothesis concerning hemochromatosis-HLA association. *Am J Hum Genet* 1987;41:89-124.
- Niederer C, Fischer R, Sonnenberg A, Stremmel W, Trampisch HJ, Stromeyer G. Survival and causes of death in cirrhotic and in non cirrhotic patients with primary hemochromatosis. *N Engl J Med* 1985;313:1256-62.
- Borwein S, Ghent CN, Valberg LS. Diagnostic efficacy of screening tests for hereditary hemochromatosis. *Can Med Assoc J* 1984;121:895-901.
- Powell LW, Summers KM, Board PG, Axelsen E, Webb S, Halliday JW. Expression of the hemochromatosis in homozygous subjects. Implication for early diagnosis and prevention. *Gastroenterology* 1990;98:1625-32.
- Simon M, Yaouanq J, Fauchet R, Le Gall JY, Brissot P, Bourel M. Genetics of hemochromatosis: HLA association and mode of inheritance. *Ann NY Acad Sci* 1988;526:11-22.
- David V, Papadopoulos P, Yaouanq J, et al. Ferritin H gene polymorphism in idiopathic hemochromatosis. *Hum Genet* 1989;81:123-6.
- David V, Boretto J, Jouanolle AM, et al. Two polymorphisms at the locus D6S98 defined by a YAC. *Nucleic Acids Res* 1990;18:5582.
- Boretto J, Jouanolle AM, Yaouanq J, et al. Anonymous markers located on chromosome 6 in the HLA class I region: allelic distribution in genetic haemochromatosis. *Hum Genet* (in press).
- Powell LW, Bassett ML, Axelsen E, Ferluga J, Halliday JW. Is all genetic (hereditary) hemochromatosis HLA-associated? *Ann NY Acad Sci* 1988;526:23-33.
- Sood AK, Pereira D, Weissman SM. Isolation and partial nucleotide sequence of cDNA clone for human histocompatibility antigen HLA-B by use of an oligodeoxynucleotide. *Proc Natl Acad Sci USA* 1981;78:616-20.
- Koller BH, Sidwell B, De Mars R, Orr HT. Isolation of HLA locus specific DNA probes from the 3' untranslated region. *Proc Natl Acad Sci USA* 1984;81:5175-8.
- Coppin HL, Denny DW, Weissman SM, McDevitt HO. HLA-B locus polymorphism: studies with a specific hybridization probe. *Proc Natl Acad Sci USA* 1985;82:8614-8.
- Cann HM, Ascanio L, Paul P, Morcadet A, Dausset J, Cohen D. Polymorphic restriction endonuclease fragment segregates and correlates with the gene for HLA-B8. *Proc Natl Acad Sci USA* 1983;80:1665-8.
- Jouanolle AM, Yaouanq J, Blayau M, et al. HLA class I polymorphism in genetic hemochromatosis. *Hum Genet* 1990;85:279-82.
- Beaudet AL, Feldman GL, Fernbach SD, Buffone GJ, O'Brien WE. Linkage disequilibrium, cystic fibrosis, and genetic counseling. *Am J Hum Genet* 1989;44:319-26.