A rapid method of haplotyping *HFE* mutations and linkage disequilibrium in a Caucasoid population

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Abstract

***Background*—*HFE* mutations are associated with hereditary haemochromatosis. However, a simple method capable of demonstrating the *cis*/*trans* arrangement of alleles is lacking, and linkage disequilibrium between *HFE* alleles and classic HLA loci is unknown. These are important issues as the pathogenic role of the mutations is not known.**

***Aims*—To develop a simple method of genotyping *HFE* mutations suitable for clinical use in addition to large disease studies.**

***Patients*—A total of 330 Caucasoid cadaveric organ donor controls were examined. Ten individuals previously *HLA-H* genotyped by polymerase chain reaction using restriction fragment length polymorphism (PCR-RFLP) were also examined to validate the method.**

***Methods*—A simple polymerase chain reaction using sequence specific primers (PCR-SSP) capable of haplotyping the mutations was developed. *HFE* allele and haplotype frequencies and linkage disequilibrium with eight HLA class I and II loci were examined in the control population.**

***Results*—27% and 19.7% of patients were positive for the 63D and 282Y alleles, respectively. No chromosome carried both 63D and 282Y. Linkage disequilibrium between 282Y and HLA-A*03 was confirmed, but was not straightforward: some A*03-associated alleles (DRB1*15, DQB1*06), but not all (B*07, Cw*0702), were associated with 282Y.**

***Conclusions*—Linkage disequilibrium data suggest that an HLA-B*07 containing haplotype contains an element affording protection from haemochromatosis and may suggest the timing of the founder 282Y mutation.**

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Keywords: *HFE*; haemochromatosis; PCR-SSP; linkage disequilibrium

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The cloning of a novel human leucocyte antigen (HLA) class I like gene, *HFE*,[1]* has generated considerable interest. Mutations in this gene have been associated with hereditary haemochromatosis[1 4 5] and porphyria cutanea tarda.[6] A single nucleotide change at codon 282 results in an amino acid substitution, Cys → Tyr (282Y [2]†). Homozygosity for 282Y is present in 90% of patients with haemochromatosis, whereas approximately 10% of controls have been found to be heterozygous.[1 4 5] Many haemochromatosis patients heterozygous for 282Y are also heterozygous for a second polymorphism at codon 63, His → Asp (63D) which is also present in approximately 25% of healthy controls.[1 5] It is unclear whether these 282Y and 63D cause haemochromatosis directly or via linkage to another mutation. Formal proof of a causal role of these mutations in haemochromatosis awaits investigation of the role of *HFE* in iron metabolism.

Several additional aspects of *HFE* polymorphism require further investigation. Haemochromatosis has long been known to be associated with HLA-A*03 as part of an extended HLA haplotype,[7] and reports thus far have confirmed the expected linkage disequilibrium between 282Y and HLA-A*03. However, there are limited data concerning the frequency of the *HFE* alleles in UK Caucasoids, and no data concerning linkage disequilibrium between these alleles and class I and II HLA genes in control populations. Whilst associations between the 282Y and 63D and haemochromatosis have been detected,[1 4 5] it is unclear whether the mutations directly induce disease or act via an association with hitherto unrecognised loci. It therefore remains of considerable interest to examine linkage disequilibrium between the *HFE* mutations and other HLA genes. For this reason we have examined associations between the *HFE* mutations and eight classic HLA class I and II loci in 330 UK Caucasoid controls.

Furthermore, there appears to be an interplay between alleles at the two *HFE* polymorphisms. For example, heterozygosity for both 282Y and 63D (compound heterozygosity) appears to confer susceptibility to haemochromatosis,[1 8] but it is important to determine whether this synergism occurs when the two mutations are present on the same (*cis*)

*There is confusion regarding this nomenclature. The term "HLA-H" was first used by Feder *et al*,[1] but was previously used to denote a distinct HLA class I-like pseudogene unrelated to haemochromatosis.[2] It has thus been recommended that the name of the gene under discussion revert to "*HFE*", the name assigned by the Genome Database Nomenclature Committee to the haemochromatosis locus.[3]

† The currently accepted nomenclature for the codon 63 His(Asp ('H63D') and 282 Cys(Tyr ('C282Y') mutations results in confusion when haplotypes are described. Thus in this paper, alleles are referred to by the codon number followed by the amino acid variant (63H, 63D, 282C and 282Y); haplotypes are denoted by each codon number followed by the amino acid allele, e.g. a haplotype of His at codon 63 andTyr at codon 282 is denoted as '63H-282Y'.

Table 1 Details of reaction mixtures used for HFE genotyping

Reaction number	*Sense primer number*	*Sense primer sequence (5'–3')*	*Nucleotide position*	*Antisense primer number*	*Antisense primer sequence (5'–3')*	*Nucleotide position*	*Specificity*	*Product size (bp)*
1	805	AGCTGTTCGTGTTCTATGATC	388–408	807	CTGGGTGCTCCACCTGGC	1066–1083	63H–282C	1460
2	805	AGCTGTTCGTGTTCTATGATC	388–408	808	CTGGGTGCTCCACCTGGT	1066–1083	63H–282Y	1460
3	806	AGCTGTTCGTGTTCTATGATG	388–408	807	CTGGGTGCTCCACCTGGC	1066–1083	63D–282C	1460
4	806	AGCTGTTCGTGTTCTATGATG	388–408	808	CTGGGTGCTCCACCTGGT	1066–1083	63D–282Y	1460
5	805	AGCTGTTCGTGTTCTATGATC	388–408	835	CTGTGGTTGTGATTTTCCATAA	535–566	63H	178
6	806	AGCTGTTCGTGTTCTATGATG	388–408	835	CTGTGGTTGTGATTTTCCATAA	535–566	63D	178
7	834	TTGGTGAAGGTGACACATCAT	846–866	807	CTGGGTGCTCCACCTGGC	1066–1083	282C	237
8	834	TTGGTGAAGGTGACACATCAT	846–866	808	CTGGGTGCTCCACCTGGT	1066–1083	282Y	237
Control primers (reactions 1–4)								
	725	GCCTTCCCAACCATTCCCTT		726	TCACGGATTTCTGTTGTGTTTC		HGH control	425

Specificity of primers is determined by the underlined 3' nucleotide. Primers 834 and 835 are consensus, non-allele specific primers. Nucleotides are numbered according to Feder *et al*[4] (Genbank accession number U60319). The genomic sequence of *HFE* has not been published, but from the known sequence of HLA-A, codons 63 and 282 would lie in exons 2 and 4, respectively, separated by about 1460 nucleotides. Examples of reactions 1–4 only are given in fig 1.

or opposite (*trans*) chromosomes. The observation that no 282Y homozygotes also carry the 63D mutation suggests that no chromosome carries both mutations and that the mutations are always in *trans* in compound heterozygotes.[8 9] However, without recourse to family studies, existing *HFE* genotyping techniques are incapable of formally demonstrating *cis*/*trans* orientation of codon 63 and 282 alleles. Current genotyping techniques require the use of restriction enzymes or labelled oligonucleotide primers or probes.[1 4 5] These techniques involve several steps, are time consuming and expensive, and often pose interpretative difficulty. Consequently we have developed a rapid, simple and reliable one step *HFE* genotyping technique using the polymerase chain reaction and sequence specific primers (PCR-SSP). Not only does this method identify alleles at each polymorphism, it also formally demonstrates haplotypes (combinations of alleles at codons 63 and 282 present on the same chromosome).

Methods

PRIMER DESIGN

Sense primers specific for the codon 63 polymorphism were combined with antisense primers specific for codon 282 to amplify all four possible *HFE* haplotypes: 63H–282C, 63H–282Y, 63D–282C, and 63D–282Y (table 1). Control primers specific for the human growth hormone (HGH) gene were used to verify successful amplification in each reaction.[10]

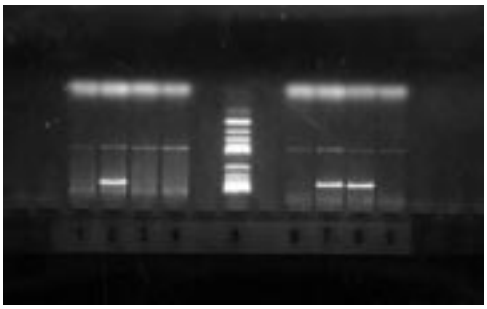

Figure 1 Examples of HFE genotyping. PCR products have been electrophoresed in ethidium bromide impregnated 1% agarose gels prior to UV illumination. The direction of electrophoresis is upwards. Each lane has an (uppermost) 425 bp control PCR product, with or without a (lower) HFE haplotype specific product of approximately 1460 bp. Lanes 1–4: haemochromatosis patient; positive lane 2, 63H–282Y homozygous. Lane 5: DNA ladder. Lanes 6–9: control sample; positive lanes 7 and 8; 63H–282Y and 63D–282C heterozygous (compound heterozygote).

DNA EXTRACTION

Genomic DNA was extracted from EDTA or tri-sodium citrate anticoagulated peripheral blood by a salting out method.[11] The buffy coat from 5 ml of anticoagulated blood was lysed in a red cell lysis buffer (RCLB) consisting of 0.144 M NH_4Cl and 1 mM $NaHCO_3$. After centrifugation the pellet was rinsed in RCLB and resuspended in 3 ml nuclear lysis buffer (0.4 M NaCl, 2 mM Na_2EDTA, 10 mM Tris-HCl) and 200 µl of 10% (w/v SDS) in water. After vigorous mixing 1 ml of 6 M NaCl and 3 ml of chloroform were added, vortexed, and centrifuged at 3000 rpm for 30 minutes. DNA aspirated from the top aqueous phase was precipitated with 95% ethanol, washed in 70% ethanol, and resuspended in distilled water.

AMPLIFICATION CONDITIONS

Reaction mixtures of 13 µl were used, consisting of 67 mM Tris base pH 8.8, 16.6 mM ammonium sulphate, 2 mM magnesium chloride, 0.01% (v/v) Tween 20, 200 mM each of dATP, dTTP, dGTP and dCTP, *HFE* forward and reverse primers at 6.8 µM each, HGH control primers at 0.68 µM, between 0.1 and 0.01 µg DNA and 0.1875 units of Taq polymerase (Advanced Biotechnology, London, UK). Reaction mixtures were dispensed under 10 µl of mineral oil (Sigma, UK) in 96-well PCR plates (Costar, High Wycombe, UK).

DNA samples were amplified in GeneAmp PCR system 9600 (Perkin-Elmer Corporation) or in MJ Research PTC-200 thermal cyclers with cycling parameters as follows: one minute at 96°C followed by five cycles of 96°C for 25 seconds, 70°C for 45 seconds, and 72°C for 45 seconds, followed by 21 cycles of 96°C for 25 seconds, 65°C for 50 seconds, and 72°C for 45 seconds, followed by four cycles of 96°C for 25 seconds, 55°C for 60 seconds, and 72°C for 120 seconds. PCR plates were sealed with Thermowell sealers (Costar) and dipped in mineral oil to improve plate to block contact.

GEL ELECTROPHORESIS AND PRODUCT DETECTION

Following PCR, 5 µl of loading buffer consisting of 0.25% Orange G, 30% v/v glycerol, and 0.5× TBE buffer (89 mM Tris base, 89 mM boric acid, 2 mM EDTA, pH 8.0) was added to

Table 2 HLA-HFE phenotype, allele and gene frequencies in 330 UK white controls

Allele	*Phenotype count (n=330)*	*Phenotype frequency*	*Allele count (n=660)*	*Allele frequency*	*Gene frequency*
63H	326	0.99	568	0.86	0.63
63D	89	0.27	92	0.14	0.07
282C	329	1.00	594	0.90	0.68
282Y	65	0.20	66	0.10	0.05

each reaction mix. PCR products (amplicons) were then electrophoresed in 1.0% agarose gels containing 0.5 µg/ml ethidium bromide for 20–25 minutes at 15 V/cm in 0.5× TBE buffer, visualised with UV illumination and photographed with a Polaroid land camera. Examples of a compound heterozygote and a 282Y homozygote are shown in fig 1.

VALIDATION

A second PCR-SSP approach was used to exclude false positive or false negative amplification. Allele specific primers for each polymorphism were used in conjunction with non-allele specific primers for four supplementary reactions (table 1, reactions 5–8). These reactions identified alleles at each polymorphism, but not haplotypes. Absolute concordance between the two methods was obtained for codon 63 and 282 genotypes in all samples.

To validate further the method for codon 282 alleles, 10 patients with suspected haemochromatosis previously genotyped for codon 282 by polymerase chain reaction using restriction fragment length polymorphism (PCR-RFLP) were also examined.

CONTROL SELECTION

A total of 330 UK Caucasoid cadaveric renal allograft donors were used as a control population. HLA-A, -B, -Cw, -DRB1, -DRB3–5, and -DQB1 genotyping was performed for all controls as previously described.[10]

DATA ANALYSIS AND STATISTICS

Mutation frequencies were determined in three ways: as phenotype frequencies (the proportion of controls positive for a mutation), allele frequencies (proportion of positive chromosomes, determined by direct counting), and gene frequencies. Gene frequencies were calculated using equation 1 where p = gene frequency and f = allele frequency:

$$p = 1-\sqrt{1-f} \qquad (1)$$

Alleles at each polymorphism were said to be in Hardy-Weinberg equilibrium if the observed homozygote and heterozygote frequencies did not differ significantly ($p>0.05$) from those expected when analysed by the χ^2 test. Linkage disequilibrium between *HFE* alleles and HLA class I and II alleles was initially assessed by χ^2 analysis of 2×2 contingency tables. Probable haplotypes thus identified were directly counted and the resulting haplotype frequencies used to calculate Δs, a measure of the difference between observed and expected haplotype frequencies (equation 2; P_{ab} = observed frequency of haplotype *ab* and P_a and P_b = frequencies of alleles *a* and *b*):

$$\Delta s = \frac{\Delta}{\Delta \max} = \frac{P_{ab} - (P_a \times P_b)}{P_a(1 - P_b)} \qquad (2)$$

Linkage disequilibrium data are also represented using the correlation coefficient r, calculated using equation 3 where n = number of observations:

$$r = \sqrt{\frac{\chi^2}{n}} \qquad (3)$$

Results

Control phenotype, allele, and gene frequencies are listed in table 2. Comparison of observed and expected genotype frequencies (table 3) demonstrated that codon 63 and 282 alleles were in Hardy-Weinberg equilibrium; 19.7% of controls carried 282Y. One control was 282Y homozygous and 11 were compound heterozygotes. Iron overload was not evident at autopsy in any of these individuals, although this was not formally excluded by serum iron studies and liver biopsy. Furthermore, it was directly shown that 63D and 282Y were in *trans* in all 11 compound heterozygotes.

Codon 282 genotype was identical by both PCR-SSP and PCR-RFLP in the 10 patients genotyped by both methods (nine patients

Table 3 HFE genotype and haplotype frequencies in 330 controls

		Observed		*Expected*		
	Genotype	*Number (total = 330)*	*Frequency*	*Number*	*Frequency*	*Hardy-Weinberg χ^2 (p)*
Codon 63	63HH	241	0.73	244	0.74	
	63HD	85	0.26	80	0.24	0.57 (0.75)
	63DD	4	0.01	6	0.02	
Codon 282	282CC	265	0.80	267	0.81	
	282CY	64	0.19	59	0.18	1.21 (0.55)
	282YY	1	0.003	3	0.01	
	Haplotype	*Number (total = 330)*	*Frequency*			
Codons 63–282	63H–282C	314	0.95			
	63D–282C	89	0.27			
	63H–282Y	65	0.20			
	63D–282Y	0	0			
Compound heterozygotes (63H–282Y and 63D–282C)		11	0.033			

Table 4 Linkage disequilibrium between HFE and HLA class I and II alleles

Haplotype	*Haplotype count*	*Haplotype frequency*	χ^2	χ^2 *p*	*Δs*	*r*
282Y–A*03	33	0.05	15.86	<0.0001	0.41	0.22
282Y–B*07	26	0.04	0.91	NS	0.26	0.05
282Y–C*0702	27	0.04	1.39	NS	0.27	0.06
282Y–DRB1*15	33	0.05	5.69	0.009	0.39	0.13
282Y–DQB1*0602	32	0.05	6.4	0.006	0.38	0.14
63D–A*29	13	0.02	6.38	0.006	0.11	0.14

Haplotype counts were obtained by counting the number of controls positive for both alleles on a haplotype. Haplotype frequencies were calculated by dividing the number of counted haplotypes by the total number of chromosomes studied (660). Haplotypes are listed as combinations of 282Y or 63D with class I or II alleles for convenience; however, data are identical for haplotypes listed as 63–282–class I/II combinations. This is because 282Y is only found with 63H and 63D with 282C—for example, the 282Y–A*03 date are identical to 63H–282Y–A*03 data. 63H and 282C are not in linkage disequilibrium with class I or II alleles because of the high frequencies of these alleles.
Δs, Δ standardised; *r*, correlation coefficient (for deviation, see text).

were 282Y homozygous, one was 282C/Y heterozygous).

Linkage disequilibrium data for 282Y and 63D with HLA class I and II alleles are presented in table 4. The expected linkage disequilibrium between 282Y and HLA-A*03 was confirmed (p<0.0001). Linkage disequilibrium was also observed between 282Y and some (DRB1*15, p = 0.009, and DQB1*0602, p = 0.006), but not all (HLA-B*07 and HLA-Cw*0702), HLA-A*03 associated alleles. 63D was in linkage disequilibrium with HLA-A*29 (p = 0.006). This has not previously been documented.

Discussion

We have developed a rapid and simple method of identifying alleles at the two known *HFE* polymorphisms, and of formally demonstrating the *cis*/*trans* orientation of alleles. Cumbersome restriction enzyme use and interpretation is avoided, and one person with one thermal cycler can comfortably genotype more than 100 samples per day. Reaction conditions are identical to those used for routine HLA class I and II genotyping,[10] so this method can easily be incorporated into HLA typing protocols for patients with suspected haemochromatosis.

The frequency of 282Y heterozygosity in the control population was 19.4%. Although higher than in previous reports,[1 12] the 282Y allele frequency (10%) is similar to that detected in other Caucasoid populations.[12] The observed allele and genotype frequencies are unlikely to be erroneous as a second method was used to confirm the genotype in every sample, and alleles were in Hardy-Weinberg equilibrium. Furthermore, the method has been validated by genotyping 10 samples previously typed by PCR-RFLP, with absolute concordance between the two methods. The representative nature of this control population is reinforced by the finding of the expected HLA class I and II control frequencies (particularly HLA-A*03 (28.4%), with which 282Y is associated). Whilst the 282Y rate of 19.4% reduces the relative risk for haemochromatosis in individuals carrying 282Y, this polymorphism remains a strong risk factor.

Twelve controls were either 282Y homozygotes or compound heterozygotes. Although none had evidence of iron overload at autopsy, it is possible that features of iron overload would have developed with time.

The expected linkage disequilibrium between 282Y and HLA-A*03 was observed, but this association was not entirely straightforward. Although HLA-A*03 was in linkage disequilibrium with HLA-B*07, -Cw*0702, -DRB1*15, and -DQB1*0602 in the control population, 282Y was only associated with A*03, DRB1*15, and DQB1*0602. HLA-B*07 and -Cw*0702 are also in linkage disequilibrium with other HLA-A alleles such as A*02. We speculate that another haplotype containing HLA-B*07 and -Cw*0702 contains an element affording protection against haemochromatosis. The presence of 282Y on the A*03-B*07-Cw*0702 haplotype, but not other B*07 haplotypes, may suggest the timing of the original "founder" 282Y mutation. This hypothesis will require testing in further studies.

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