Immunogenetics (1998) 47: 404–410

ORIGINAL PAPER

**Graça Porto · Helena Alves · Pedro Rodrigues
José M Cabeda · Cristina Portal · Anunciação Ruivo
Benvindo Justiça · Roger Wolff · Maria De Sousa**

Major histocompatibility complex class I associations in iron overload: evidence for a new link between the *HFE* H63D mutation, *HLA-A29*, and non-classical forms of hemochromatosis

Received: 11 June 1997 / Revised: 29 October 1997

Abstract The present study is an analysis of the frequencies of *HFE* mutations in patients with different forms of iron overload compared with the frequencies found in healthy subjects from the same region. The frequencies of HLA-A and -B antigens and *HLA* haplotypes were also analyzed in the same subjects. The study population included: 71 healthy individuals; 39 genetically and clinically well-characterized patients with genetic hemochromatosis (HH); and 25 patients with non-classical forms of iron overload (NCH), excluding secondary hemochromatosis. All subjects were *HLA*-typed and *HFE*-genotyped by the oligonucleotide ligation assay (OLA). The gene frequencies found for the C282Y and H63D mutations of *HFE* were respectively: 0.03 and 0.23 in healthy individuals, 0.86 and 0.04 in HH patients, and 0.08 and 0.48 in NCH patients. An expected significant association between HH and HLA-A3 was observed, which was found to be in linkage disequilibrium with the C282Y mutation. A new association was seen, however, between HLA-A29 and NCH, in linkage disequilibrium with the H63D mutation. Again as expected, the HLA-B antigen B7 was associated with HH in linkage disequilibrium with HLA-A3. In addition, the HLA-B antigen B44 was found to be associated with NCH but not in linkage disequilibrium with either A29 or the H63D mutation. In conclusion, a new association of the *HFE* H63D mutation with forms of hemochromatosis other than HH and a new association between the HLA phenotype A29 and the *HFE* H63D mutation were found in the same patients. These findings reinforce evidence for the involvement of the major histocompatibility class I in iron metabolism, supporting the notion of a physiological role for the immunological system in the regulation of iron load.

Key words Iron · Hemochromatosis · MHC · HLA · *HFE*

G. Porto · J.M. Cabeda · C. Portal · A. Ruivo
Santo António General Hospital, Largo do Prof. Abel Salazar, no.1, P-4050 Porto, Portugal

G. Porto (✉) ·P. Rodrigues · J.M. Cabeda · B. Justiça · M. De Sousa
Abel Salazar Institute for the Biomedical Sciences, Largo do Prof. Abel Salazar, no. 2, P-4050 Porto, Portugal

H. Alves
North Histocompatibility Center, Lusotransplante, Rua Dr. Roberto Frias, P-4200 Porto, Portugal

R. Wolff
Mercator Genetics Inc., 4040 Campbell Avenue, Menlo Park, CA 94025, USA

Introduction

The genetic basis of hemochromatosis (HH) has been known for more than 20 years, following the demonstration of the association between the major histocompatibility complex (MHC) class I antigen HLA-A3 and the disease (Edwards et al. 1977; Simon et al. 1976). These and subsequent studies of the strong linkage disequilibrium between HH and various markers in the MHC region all led to the expectation that a putative *HH* gene would lie within approximately 1 cM of the *HLA-A* locus (Gasparini et al. 1993; Jazwinska et al. 1993; Raha-Chowdhury et al. 1995; Yaouanq et al. 1994). While the *HLA* association has had profound implications both for the clinical diagnosis and cloning strategies of the *HH* gene, the possible biological significance of that association is only now becoming evident. Two major recent developments strongly support the implication of the MHC class I in the pathogenesis of hemochromatosis. The first was the demonstration that β_2-microglobulin (β_2M)-deficient mice, which lack MHC class I molecules, display a progressive hepatic iron overload similar to that observed in human HH (De Sousa et al. 1994; Rothenberg and Voland 1996; Santos et al. 1996). The second was the identification by Feder and co-workers (1996) of the candidate gene for *HH,* termed *HFE*. This is a new MHC class I-like gene, where homozygosity for a single base pair mutation (C282Y), resulting in a cystein to tyrosine substitution at position 282, was found in 83% of HH patients. This finding was promptly confirmed by other groups who described the presence of the C282Y mutation in varying, but always very high, numbers of hemochro-

Table 1 Clinical characterization of the 20 NCH patients

Patient #	Sex	Age (Years)	Transferrin saturation (%)	Serum ferritin (ng/ml)	Hepatic iron index (Perls grade)	Iron removed (g)	Report to family	Clinical conditions associated	HLA A and B phenotype	HFE genotype
1	Male	48	56	2446	>6 (IV)	n.a.	Proband	Hereditary xerocytosis	A 29,2; B 12,35	C282Y(–/–)H63D(+/+)
2	Male	39	92	1500	>6 (IV)	9.8	Sibling	Hereditary xerocytosis	A 29,2; B 14,35	C282Y(–/–)H63D(+/–)
3	Male	37	78	2240	(III)	8.6	Proband	Haemolytic anaemia	A 2,9; B 27,51	C282Y(–/–)H63D(+/–)
4	Male	25	85	810	2.2	2.4	Proband	Haemophilia A	A 29,X; B 38,44	C282Y(–/–)H63D(+/+)
5	Male	36	81	811	4.0	1.8	Nephew	Haemophilia A	A 29,X; B 38,27	C282Y(–/–)H63D(+/+)
6	Male	60	100	3000	2.8	n.a.	Proband	Chronic hepatitis C	A 29,2; B 7,51	C282Y(–/–)H63D(+/–)
7	Male	64	92	752	(III)	2.0	Proband	Chronic hepatitis C	A 24,28; B 51,44	C282Y(–/–)H63D(+/–)
8	Male	44	78	605	3.4	1.5	Proband	Chronic hepatitis C	A 2,26; B 27,44	C282Y(–/–)H63D(–/–)
9	Male	59	60	714	(III)	2.6	Proband	Chronic hepatitis B	A 29,2; B 51,40	C282Y(–/–)H63D(+/+)
10	Male	68	87	509	2.6	3.2	Proband	Chronic hepatitis B	A 26,33; B 7,14	C282Y(–/–)H63D(+/+)
11	Male	30	62	620	(III)	2.9	Proband	Chronic hepatitis B	A 3,24; B 35,X	C282Y(–/–)H63D(–/–)
12	Male	44	57	527	(III)	3.7	Proband	Alcohol abuse+Chr. hepatitis B	A 10,11; B 14,12	C282Y(–/–)H63D(–/–)
13	Male	57	65	525	1.6	4.2	Proband	Alcohol abuse	A 29,1; B 8,44	C282Y(–/–)H63D(+/–)
14	Male	49	51	412	(III)	3.0	Proband	Alcohol abuse	A 12,X; B 35,12	C282Y(–/–)H63D(–/–)
15	Female	55	60	1254	1,5 (III)	5.3	Proband	Obesity+steatohepatitis	A 29,2; B 35,12	C282Y(–/–)H63D(+/+)
16	Male	58	53	907	2.2	4.0	Proband	Obesity	n.a.	C282Y(–/–)H63D(+/–)
17	Male	28	50	652	2.9	3.2	Son		n.a.	C282Y(–/–)H63D(+/–)
18	Male	31	68	362	4.8	2.3	Proband		A 29,28; B 37,X	C282Y(+/–)H63D(+/–)
19	Male	28	27	144	(II)	n.a.	Proband		A 3,30; B 7,14	C282Y(+/–)H63D(+/–)
20	Male	25	22	281	(II)	n.a.	Sibling		A 3,11; B 7,37	C282Y(–/–)H63D(+/–)
21	Male	44	47	438	2.0	1.9	Proband		A 2,11; B 44,X	C282Y(+/–)H63D(–/–)
22	Male	34	30	563	2.6	2.1	Proband		A 1,X; B 8,X	C282Y(–/–)H63D(+/+)
23	Male	46	59	773	1.4 (II)	n.a.	Proband		A 24,28; B 44,X	C282Y(–/–)H63D(–/–)
24	Male	44	41	1000	(III)	3.5	Proband		n.a.	C282Y(–/–)H63D(+/–)
25	Male	50	39	821	2.0	2.6	Sibling		n.a.	C282Y(–/–)H63D(+/–)

matosis patients in several parts of the world (Barton et al. 1997; Beutler et al. 1996; Borot et al. 1997; Carella et al. 1997; Jazwinska et al. 1996; Jouanolle et al. 1996). A second mutation was also found in the *HFE* gene (H63D) resulting in a histidine to aspartic acid substitution at position 63. This variant was found to be highly frequent in normal chromosomes (17%) (Feder et al. 1996) but its contribution to HH is less clear.

From the clinical point of view, identification of the *HFE* mutations is of great relevance. It not only allows a better screening of HH but it provides the opportunity to characterize more precisely other iron overload syndromes where the *HH* gene implication has always been controversial. These include forms of hemochromatosis associated with other hematological disorders, chronic alcoholic or viral hepatitis, or the new syndrome of iron overload with normal transferrin saturation recently described by Moirand and co-workers (1997). The lack of association of the HLA-A3 phenotype in those forms of iron overload has been classically used as an argument supporting the non-HLA linkage of the disease. To our knowledge, however, associations with other HLA phenotypes or with *HFE* have not yet been demonstrated. The same is true for the African dietary iron overload, where a still unknown genetic factor has been identified (Gordeuk et al. 1992).

In this study, the frequencies of *HFE* mutations in a group of patients with several forms of non-transfusional iron overload are compared with the frequencies found in HH patients and healthy subjects from the same geographical area. Looking for possible interactions between HLA phenotypes and *HFE* mutations, we estimated the frequencies of various HLA-A and -B antigens and *HLA* haplotypes in the same groups of subjects.

Materials and methods

Patients

A total of 64 patients with iron overload were included in this study. Of those patients, 39 (25 unrelated probands and 14 family members) were genetically and clinically well-characterized HH patients according to previously described criteria (Porto et al. 1997), and 25 (20 unrelated probands and 5 family members) were diagnosed as hemochromatosis patients based on biochemical and histological grounds but who did not fulfill the criteria to be classified as having genetic hemocromatosis, either because they had other associated clinical conditions (hematological disorders, chronic alcoholic or viral liver disease, disturbance of lipid metabolism), and/or showed lower levels of iron stores (<5 g) estimated by quantitative phlebotomies. These patients were classified as having non-classical hemochromatosis (NCH). The most relevant clinical data in the group of NCH patients are summarized in Table 1. These patients constituted a clinically heterogeneous group but had the common characteristic of excessive iron deposits in parenchymal cells demonstrated by liver biopsy and/or an hepatic iron index greater than 2, making difficult the differential diagnosis with early forms of HH. Two main characteristics, besides the presence of associated clinical conditions, may distinguish the NCH group from HH probands: 1. The level of total body iron stores is usually in the range of 2–4 g (see

Table 2 Allele frequencies of HLA-A antigens in the two groups of patientswith iron overload (FAD1 and FAD2) in comparison with the control group (FAP)

	HH Patients				Patients with NCH				Controls
HLA	FAD1 (*n = 48*)	*RR*	δ	*P*	FAD2 (*n* = 36)	*RR*	δ	*P*	FAP (*n = 142*)
A1	0.10	1.00	0.00	n. s.	0.06	0.57	–0.04	n. s.	0.10
A2	0.15	0.48	–0.16	n. s.	0.22	0.76	–0.07	n. s.	0.27
A3	***0.44***	***9.04***	***0.39***	***4.01E-8***	0.06	0.73	–0.02	n. s.	0.08
A9	0.17	1.50	0.06	n. s.	0.08	0.64	–0.02	n. s.	0.12
A10	0.02	0.18	–0.09	n. s.	0.08	0.78	–0.02	n. s.	0.10
A11	0.02	0.23	–0.07	n. s.	0.06	0.73	–0.02	n. s.	0.08
A28	0.02	0.66	–0.01	n. s.	0.08	2.81	0.05	n. s.	0.03
A29	–	0.00	–0.06	n. s.	***0.22***	***4.42***	***0.17***	***3.78E-3***	0.06
A32	0.02	2.90	0.01	n. s.	–	0.00	–0.01	n. s.	0.007
A33	–	0.00	–0.02	n. s.	0.03	1.52	0.01	n. s.	0.02

RR = Relative risk
δ = Etiologic fraction
n = Total number of alleles in each group
P = Level of significance of the associations
n. s. = Not significant

Table 1), in marked contrast with HH where a level of iron stores greater than 5 g was used as a diagnostic criterion (the range in the present group of HH probands was 5–13 g). 2. The transferrin saturation, which is a homogeneous biochemical marker of iron overload in HH patients (ranging from 60% to 110% in the present group of HH patients), showed some heterogeneity in the NCH group. Seven patients in the present series had normal transferrin saturation (<50%) with unexplained hepatic iron overload and hyperferritinemia (see Table 1). These were clinically related to those described by Moirand and co-workers (1997) with the new syndrome of iron overload with normal transferrin saturation. In this study we did not include patients with secondary forms of iron overload such as blood transfusion.

A group of 71 unrelated apparently healthy subjects from the same geographical area in the north of Portugal was used as a control population. None of the controls had evidence of iron overload in terms of biochemical parameters. All participants in this study gave their informed consent.

C282Y and H63D mutation detection

HFE genotyping was performed by OLA on polymerase chain reaction (PCR)-amplified genomic DNA samples using amplification primers for the C282Y or H63D mutations and the normal allele as previously described (Feder et al. 1996).

HLA-A and -B typing

HLA typing was performed in freshly collected venous blood samples by the standard complement-dependent micro-lymphocytotoxicity assay using a battery of sera which enabled the definition of the following HLA antigens: *A*-locus: 1, 2, 3, 9 (23,24,25), 10 (26,34), 11, 28, 29, 32 and 33; *B*-locus: 5 (51), 7, 8, 12 (44,45), 13, 14, 15 (62), 16 (38), 17 (57), 18, 21 (49,50), 22, 27, 35, 37, 40, 70.

Family studies and haplotype analysis

Family studies for the screening of hemochromatosis were done systematically in all first-degree relatives of all patients and included *HLA* typing. For most of those relatives, frozen material stored at the time of the study was available, permitting the present detection of the *HFE* mutations. Segregation analysis was then performed, allowing in most cases the assignment of the *HLA* haplotypes as well as the definition of the *HLA* haplotypes associated with the *HFE* mutations.

Statistics

In all statistical analyses, patients were divided into two groups according to the clinical presentation: HH patients and those with NCH. Allele frequencies were estimated for the *HFE* mutations and HLA antigens in the two groups of patients and compared with the same frequencies in the control population. For those estimations, only unrelated probands were included. Whenever a single HLA antigen was defined in one subject and the family study could not check the segregation, its frequency was not taken into account in calculating the value for the blank allele. If, on the other hand, family studies showed segregation in two different haplotypes, it was taken as the specificity of the blank. To analyze the relative strength in disease association of the different *HFE* or *HLA* alleles, the etiological fraction δ was calculated as described according to the formula *d*= (FAD-FAP)/(1-FAP), where FAD is the allele frequency in the diseased population and FAP the allele frequency in the control population (Bengtsson and Thomson 1981; Thomson et al. 1983). The same analysis was done for haplotype frequencies where FHD is the frequency of the haplotype in the diseased population. Since no family studies were done in controls and, therefore, no haplotypes were defined, we estimated the range of δ values [δ] for haplotype associations by comparing the FHD with the range of frequencies in the control population [FHP]. This range may vary from zero, where the pairs of alleles in question are assumed to be always in different chromosomes, to the maximum frequency estimated assuming that the alleles in question are always in the same chromosome. Comparisons between frequencies in different groups were done by the chi-square test with Yates' correction for small samples.

Results

HFE and HLA allele frequencies

Of the 25 unrelated HH patients (21 males and 4 females), all carried at least one of the described *HFE* mutations: 21 were found homozygous for the C282Y mutation, two were heterozygous, one was a double heterozygote for the C282Y and the H63D mutations, and one was a heterozygote for the H63D mutation only. This corresponds to gene frequencies of 0.86 for the C282Y and 0.04 for the

Table 3 Allele frequencies of HLA-B antigens in the two groups of patients with iron overload (FAD1 and FAD2) in comparison with the control group (FAP)

	HH Patients				Patients with NCH				Controls
HLA	FAD1 (*n = 48*)	*RR*	δ	*P*	FAD2 (*n = 36*)	*RR*	δ	*P*	FAP (*n = 142*)
B5	0.08	0.78	–0.02	n. s.	0.11	1.11	0.01	n. s.	0.10
B7	***0.29***	***7.76***	***0.25***	***3.66E-6***	0.08	1.65	0.03	n. s.	0.05
B8	0.08	1.16	0.01	n. s.	0.06	0.85	–0.01	n. s.	0.07
B13	0.02	2.90	0.01	n. s.	–	0.00	–0.01	n. s.	0.007
B14	0.02	0.23	–0.07	n. s.	0.08	1.00	0.00	n. s.	0.08
B15	0.02	0.49	–0.02	n. s.	–	0.00	–0.04	n. s.	0.04
B16	–	0.00	–0.02	n. s.	0.03	1.52	0.01	n. s.	0.02
B17	0.04	0.55	–0.04	n. s.	–	0.00	–0.08	n. s.	0.07
B18	–	0.00	–0.04	n. s.	–	0.00	–0.04	n. s.	0.04
B21	0.04	0.65	–0.02	n. s.	–	0.00	–0.06	n. s.	0.06
B22	–	0.00	–0.02	n. s.	–	0.00	–0.02	n. s.	0.02
B27	0.04	0.31	–0.10	n. s.	0.06	0.47	–0.07	n. s.	0.12
B35	0.04	0.48	–0.05	n. s.	0.11	1.42	0.02	n. s.	0.08
B37	–	0.00	–0.01	n. s.	0.03	4.39	0.02	n. s.	0.007
B40	0.02	0.07	–0.01	n. s.	0.03	1.00	0.00	n. s.	0.03
B44	0.10	0.74	–0.05	n. s.	***0.31***	***3.01***	***0.21***	***3.10E-3***	0.13
B45	–	0.00	–0.01	n. s.	–	0.00	–0.01	n. s.	0.01
B70	–	0.00	–0.01	n. s.	–	0.00	–0.01	n. s.	0.007

RR = Relative risk
δ = Etiologic fraction
n = Total number of alleles in each group
P = Level of significance of the associations
n. s. = Not significant

H63D mutations, frequencies not different from those previously reported in other HH populations. Of the 71 healthy individuals, four were found heterozygous for the C282Y mutation, five were homozygous, and 22 heterozygous for the H63D mutation; all other subjects carried neither mutation. This corresponds to gene frequencies of 0.03 and 0.23 for the C282Y and H63D mutations, respectively, again similar to the previously reported results in a healthy USA population (Feder et al. 1996). Of the 20 unrelated NCH patients (19 males and 1 female): one was heterozygous for the C282Y mutation, two were compound heterozygotes, six were homozygous and five heterozygous for the H63D mutation, and six carried neither mutation. This corresponds to a gene frequency of 0.08 for the C282Y mutation (significantly lower than in classical HH) and an unexpectedly high frequency of 0.48 for the H63D mutation.

The allele frequencies of the HLA antigens found in the two groups of patients are listed in Tables 2 and 3 in comparison with the same frequencies in the control population. As expected, significant associations were found for HLA antigens A3 and B7 in HH patients, the association of A3 being stronger than B7 but none of them stronger than the association with the C282Y mutation (see δ values in Tables 2–4). The frequency of the C282Y mutation in HH patients with the HLA A3 and/or B7 phenotypes was 1.00, confirming the strong linkage disequilibrium between the three loci. In patients with NCH, significant associations were found for HLA antigens A29 and B44. Again, none of them was stronger than the association with the H63D mutation (see δ values in Tables 2–4). In order to test whether a linkage disequilibrium also existed among H63D, A29, and B44, the H63D mutation frequencies were compared in patients and controls with the HLA phenotypes A29 or B44. Of a total of 17 subjects with the HLA phenotype A29 (7 NCH patients and 10 controls), 13 (7 patients and 6 controls) had the H63D mutation. This frequency (76%) was significantly higher ($P < 0.002$) than those found in NCH patients or controls lacking the HLA A29 phenotype (36% and 34%, respectively). In contrast, the frequencies of appearance of the H63D mutations in patients or controls with the phenotype B44 were not significantly different from those in subjects without the B44 phenotype. Although a linkage disequilibrium between A29 and B44 has been described in the Portuguese population as well as in other European Caucasoid populations (Silva Carvalho 1983), in the present groups of subjects we could not confirm the existence of such linkage. The fact that the present control population comes from a geographically restricted area in the north of Portugal could be influencing this result. To further test the existence of linkage disequilibria between the *HFE* mutations and *HLA* alleles, we proceeded with the analysis of haplotype associations in both groups of patients.

Haplotype analysis

The segregation analysis done with family studies permitted the definition of 76 *HLA* haplotypes: 42 in HH

Table 4 Allele frequencies of HFE mutations in the two groups of patients with iron overload (FAD1 and FAD2) in comparison with the control group (FAP)

	HH Patients				Patients with NCH				Controls
HFE	FAD1 (*n = 50*)	*RR*	δ	*P*	FAD2 (*n = 40*)	*RR*	δ	*P*	FAP (*n = 142*)
C282Y	***0.86***	***198.62***	***0.86***	***0.00000***	0.08	2.81	0.05	n. s.	0.03
H63D	0.04	0.14	–0.25	4.10E-3	***0.48***	***3.09***	***0.32***	***3.38E-4***	0.23

RR = Relative risk
δ = Etiologic fraction
n = Total number of alleles in each group
P = Level of significance of the associations
n. s. = Not significant

Table 5 Frequencies of the relevant haplotype combinations in HH or NCH patients (FHD) in comparison with their frequency ranges in normal controls [FHP]. For comparison are also indicated the frequencies and δ values of the mutations H63D and C282Y in the respective groups

		FHD (*n = 34*)	[FHP] (*n = 142*)	[δ]	*P*
	H63D	***0.48***	***0.23***	***0.32***	***3.38E-4***
NCH Patients	H63D/A29	0.21	0–0.04	0.18–0.21	1.28E-4
	H63D/B44	0.18	0.02–0.07	0.12–0.16	n. s.
	A29/B44	0.12	0–0.03	0.09–0.12	n. s.
	H63D/A29/B44	"	"	"	"
		(*n = 42*)	(*n = 142*)		
	C282Y	***0.86***	***0.03***	***0.86***	***0.00000***
HH Patients	C282Y/A3	0.45	0.007	0.446–0.450	3.33E-16
	C282Y/B7	0.26	0–0.007	0.19–0.26	4.19E-9
	A3/B7	0.19	0.000	0.19	3.77E-15
	C282Y/A3/B7	"	"	"	"

[δ] = Etiologic fraction range
P = Significance level of the associations
n. s. = Not significant
n = Total number of haplotypes defined in each group

patients (36 from the probands and 6 additional from other affected family members) and 34 in NCH patients (31 from the probands and 3 additional from other affected family members). All except one of the 42 haplotypes defined in HH patients segregated with the C282Y mutation. Among these, A3/B7 was the most common with a haplotype frequency of 0.19. Of the 34 haplotypes defined in NCH patients, 17 segregated with the H63D mutation. Among these, A29/B44 was the most common with a haplotype frequency of 0.12. The δ values for the different combinations of the most frequent alleles in the two groups are shown in Table 5. The simple comparison of those δ values shows that in both forms of hemochromatosis the strongest associations occur with the *HFE* mutations alone (C282Y and H63D, respectively, in HH and NCH). Significant associations were observed between the C282Y or H63D mutations and the HLA antigens A3 or A29, respectively (see Table 5), reflecting the strong linkage disequilibrium between the *HFE* and *HLA-A* loci. The linkage between *HLA-A* and *-B* loci was also observed for A3 and B7 in HH patients but it was not significant for A29 and B44 in NCH patients. This result contrasts with the significant allelic association of B44 alone in the same patients (see Table 3).

Discussion

It is becoming increasingly evident that the clinical spectrum of iron overload disorders is changing. Early forms of unexpressed HH are much more common, making the differential diagnosis with other less severe forms of iron overload difficult. The recent description by Moirand and co-workers (1997) of a new syndrome of iron overload with lower iron stores is of clinical relevance and raises an important question about the mechanisms which may lead to parenchymal iron loading in the presence of normal plasma transferrin saturation. As well as that particular syndrome, other clinical situations exist where the pathogenesis of iron overload remains unclear. They include forms of hemochromatosis associated with other hematological disorders or chronic liver disease, in which parenchymal cell iron accumulation is found in the liver biopsy. The present findings of an association between the *HFE* H63D mutation with those iron overload conditions may represent one step forward in the search for a common pathogenic basis in all forms of iron overload, possibly in close association with MHC class I molecules.

The strong association observed between the HLA-A3 and -B7 phenotypes and the C282Y mutation was not surprising, given the well established linkage disequilibrium between those antigens and HH. A new association of HLA-A29 with the H63D mutation was found, however, further supporting a strong linkage disequilibrium between the *HFE* and *HLA-A* loci. The biological significance of that association with non-classical forms of iron overload is still not clear. Two alternative explanations could be put forward: 1. The H63D/A29 association could be a marker of a still unidentified cellular iron loading gene that would predispose to a different (usually less severe) form of iron overload, most commonly manifested when other clinical conditions were associated, such as alcoholic or viral disease. Under this assumption, the *H63D/A29* haplotype could correspond to an ancestral haplotype marking that putative gene, similarly to the haplotype *C282Y/A3/B7* in classical HH. 2. An alternative hypothesis could be that the MHC complex itself would influence iron transport either directly or indirectly through the presentation of particular viral peptides or other endogenous peptides. Within this perspective, some particular MHC configurations could constitute additional susceptibility markers of iron overload besides the *HFE* or any other putative iron loading gene, thus providing an explanation for the observed strong association of B44 with NCH independently of the H63D/A29 association.

If the MHC is either directly or indirectly involved in iron metabolism, one could further speculate that, in human evolution, some particular HLA antigens or haplotype combinations could be positively selected to protect against iron deficiency in populations with poor iron availability, as proposed earlier by Jancovic and co-workers (1989), for the haplotype A3/B7 linked to the hemochromatosis gene.

Whether the H63D/A29 association merely represents a bystander marker or has some functional role in iron overload is not determined in the present analysis but requires further investigation. It may be anticipated that the inclusion of the characterization of T lymphocytes in these patients may help to elucidate this association. Peripheral $CD8^+$ T lymphocytes are known to be activated and their numbers to be set in the context of MHC class I molecules (Freitas and Rocha 1997; Tanchot et al. 1997). Abnormally low numbers of those cells have been found in $HLA\text{-}A3^+$ HH patients with the most severe forms of iron overload (Porto et al. 1997), and the same patients also showed abnormalities in the TCR repertoire of $CD8^+$, but not $CD4^+$ T cells (Cabeda et al. 1995).

In conclusion, the present data, showing an association of the H63D mutation with forms of hemochromatosis other than HH and a new association of the HLA phenotype A29 with the H63D mutation in the same patients, reinforces evidence for MHC class I involvement in iron metabolism and supports the notion that the immunological system has a physiological role in the regulation of iron load (De Sousa 1989; De Sousa et al. 1992).

Acknowledgments For their invaluable technical assistance, we gratefully acknowledge all the laboratory staff of the Clinical Chemistry Laboratory in Santo António General Hospital and the North Histocompatibility Centre, and especially Rosa Lacerda from the Abel Salazar Institute for the Biomedical Sciences. We also acknowledge all clinicians who referred to us their hemochromatosis patients. This work was funded by JNICT grant PECS/C/SAU/59/95.

References

Barton, J.C., Shih, W.H., Sawada-Hirai, R., Acton, R.T., Harmon, L., Rivers, C., and Rothenberg, B.E. Genetic and clinical description of hemochromatosis probands and heterozygotes: evidence that multiple genes linked to the major histocompatibility complex are responsible for hemochromatosis. *Blood Cells Mol Dis 30:* 135–145, 1997

Bengtsson, B.O. and Thomson, G. Measuring the strength of associations between HLA antigens and diseases. *Tissue Antigens, 18:* 356–363, 1981

Beutler, E., Gelbart.T., West, C., Lee, P., Adams, M., Blackstone, R., Pockros, P., Kosty, M., Venditti, C.P., Pradyumna, D.P., Seese, N.K., Chorney, K.A., Ten Elshof, A.E., Gerhard, G.S., and Chorney, M. Mutation analysis in hereditary hemochromatosis. *Blood Cells Mol Dis 22:* 187–194, 1996

Borot, N., Roth, M.P., Malfroy, L., Demangel, C., Vinel, J.P., Pascal, J.P., and Copin, H. Mutations in the MHC class I-like candidate gene for hemochromatosis in French patients. *Immunogenetics 45:* 320–324, 1997

Cabeda, J.M., Porto, G., Lacerda, R., Jeddi-Tehranni, M., Silva, B.M., Gigliotti, D., Hultcratz, R., Andersson, R., Wigzell, H., and De Sousa, M. Anomalies of the CD8+ population in genetic hemochromatosis: characterization of the T-cell-receptor repertoire (abstract). *FASEB J 9:* 3051, 1995

Carella, M., D'Ambrosio, L., Totaro, A., Grifa, A., Valentino, M.A., Piperno., A., Girelli, D., Roetto, A., Franco, B., Gasparini, P., and Camaschella, C. Mutation analysis of the HFE gene in Italian hemochromatosis patients. *Am J Hum Genet 60:* 828–832, 1997

De Sousa, M. The immunology of iron overload. *In* M. De Sousa and J. Brock (eds): *Iron in Immunity, Cancer and Inflamation*, pp. 247–258, Wiley, Chichester, 1989

De Sousa, M. T lymphocytes and iron overload: new correlation of possible significance to the biology of the immunological system. *Mem Inst Oswaldo Cruz 87 [Suppl V]:* 23–29, 1992

De Sousa, M., Reimão, R., Lacerda, R., Hugo, P., Kaufmann, S.H.E., and Porto, G. Iron overload in β_2-microglobulin-deficient mice. *Immunol Lett 39:* 105–111, 1994

Edwards, C.Q., Carroll, M., Bray, P., and Cartwright, G.E. Hereditary hemochromatosis: diagnosis in siblings and children. *N Engl J Med 297*: 7–13, 1977

Feder, J.N., Gnirke, A., Thomas, W., Tsuchihashi, Z., Ruddy, D.A., Basava, A., Dormishian, F., Domingo, R., Ellis, M.C., Fullan, A., Hinton, L.M., Jones, N.L., Kimmel, B.E., Kronmal, G.S., Lauer, P., Lee, V.K., Loeb, D.B., Mapa, F.A., McClelland, E., Meyer, N.C., Mintier, G.A., Moeller, N., Moore, T., Morikang, E., Prass, C.E., Quintana, L., Starnes, S.M., Schatzman, R.C., Brunke, K.J., Drayna, D.T., Risch, N.J., Bacon, B.R., and Wolff, R.K. A new MHC class I-like gene is mutated in patients with hereditary hemochromatosis. *Nat Genet 13:* 399–408, 1996

Freitas, A. and Rocha, B. Lymphocyte survival: a red queen hypothesis. *Science 277:* 1950, 1997

Gasparini, P., Borgato, L., Piperno, A., Girelli, D., Olivieri, O., and Gottardi, E. Linkage Analysis of 6p21 polymorphic markers and the hereditary hemochromatosis: localization of the gene centromeric to HLA-F. *Hum Mol Genet 2:* 571–576, 1993

Gordeuk, V., Mukiibi, J., Hasstedt, S.J., Samowitz, W., Edwards, C.Q., West, G., Ndambire, S., Emmanual, J., Nkanza, N., Chapanduka, Z., Randall, M., Boone, P., Romano, P., Martell, R.W., Yamashita, T., Effler, P., and Brittenham, G. Iron overload in Africa. Interaction between a gene and dietary iron content. *N Engl J Med 326:* 95–100, 1992

Jancovic, G.M., Colovic, M.D., Petrovic, M.D., Suvajdzic, N., Bogdanovic, G., Radovacevic, R., Tomasevic, R., and Trpinac, D.P. Selective advantage for females with the h-allele? *Eur J Haematol 43:* 265–266, 1989

Jazwinska, E.C., Lee, S.C., Webb, S.I., Halliday, J.W., and Powell, L.W. Localization of the hemochromatosis gene close to D6S105. *Am J Hum Genet 53:* 347–352, 1993

Jazwinska, E.C., Cullen, L.M., Busfield, F., Pyper, W.R., Webb, S.I., Powell, L.W., Morris, C.P., and Wash, T.P. Hemochromatosis and HFE. *Nat Genet 14:* 249–251, 1996

Jouanolle, A.M., Gandon, G., Jézéquel, P., Blayau, M., Campion, M.L., Yaouanq, J., Mosser, J., Fergelot, P., Chauvel, B., Bouric, P., Carn, G., Andrieux, N., Gicquel, I., Le Gall, J-Y., and David, V. Hemochromatosis and HFE. *Nat Genet 14:* 251–252, 1996

Moirand, R., Mortaji, A.M., Loreal, O., Paillard, F., Brissot, P., and Deugnier, Y. A new syndrome of liver iron overload with healthy transferrin saturation. *Lancet 349:* 95–97, 1997

Porto, G., Vicente, C., Teixeira, M.A., Martins, O., Cabeda, J.M., Lacerda, R., Gonçalves, C., Fraga, J., da Silva, B.M., Alves, H., Justiça, B., and de Sousa, M. Relative impact of HLA phenotype and CD4-CD8 ratios on the clinical expression of hemochromatosis. *Hepatology 25:* 397–402, 1997

Raha-Chowdhury, R., Bowen, D.J., Stone, C., Pointon, J.J., Terwilliger, J.D., Shearman, J.D., Robson, J.D., Bonford, A., and Worwood, M. New polymorphic microsatellite markers place the hemochromatosis gene telomeric to D6S105. *Hum Mol Genet 4:* 1869–1874, 1995

Rothenberg, B.E. and Voland, J.R. B2 knockout mice develop parenchymal iron overload: a putative role for class I genes of the major histocompatibility complex in iron metabolism. *Proc Natl Acad Sci USA 93:* 1529–1534, 1996

Santos, M., Schilham, M.W., Rademakers, L.H.P.M., Marx, J.J.M., De Sousa, M., and Clevers, H. Defective iron homeostasis in β_2-microglobulin knockout mice recapitulates Hereditary Hemochromatosis in man. *J Exp Med 184:* 1975–1985, 1996

Silva Carvalho, C. A. HLA-A,B and C markers in the Portuguese population. *Tissue Antigens 21:* 39–44, 1983

Simon, M., Bourel, M., Fauchet, R., and Genetet, B. Association of HLA-A3 and HLA-B14 antigens with idiopathic hemochromatosis. *Gut 17:* 332–334, 1976

Tanchot, C., Lemonnier, F.A., Pérarnau, B., Freitas, A., and Rocha, B. Differential requirements for survival and proliferation of CD8 naïve or memory T cells. *Science 276:* 2057–2062, 1997

Thomson, G., Motro, U., and Selvin, S. Statistical aspects of measuring the strength of association between HLA antigens and diseases. *Tissue Antigens 21:* 320–328, 1983

Yaouanq, J., Perichon, M., Chorney, M., Pontarotti, P., Le Treut, A., El Kahloun, A., Mauvieux, V., Blayau, M., Jouanolle, A.M., Chauvel, B., Moirand, R., Nouel, O., Le Gall, J.Y., Feingold, J., and David, V. Anonymous marker loci within 400 kb of HLA-A generate haplotypes in linkage disequilibrium with the hemochromatosis gene (HFE). *Am J Hum Genet 54:* 252–263, 1994