

# Analysis of HLA-A antigens and C282Y and H63D mutations of the HFE gene in Brazilian patients with hemochromatosis

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## Abstract

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The hemochromatosis gene, HFE, is located on chromosome 6 in close proximity to the HLA-A locus. Most Caucasian patients with hereditary hemochromatosis (HH) are homozygous for HLA-A3 and for the C282Y mutation of the HFE gene, while a minority are compound heterozygotes for C282Y and H63D. The prevalence of these mutations in non-Caucasian patients with HH is lower than expected. The objective of the present study was to evaluate the frequencies of HLA-A antigens and the C282Y and H63D mutations of the HFE gene in Brazilian patients with HH and to compare clinical and laboratory profiles of C282Y-positive and -negative patients with HH. The frequencies of HLA-A and C282Y and H63D mutations were determined by PCR-based methods in 15 male patients (median age 44 (20-72) years) with HH. Eight patients (53%) were homozygous and one (7%) was heterozygous for the C282Y mutation. None had compound heterozygosity for C282Y and H63D mutations. All but three C282Y homozygotes were positive for HLA-A3 and three other patients without C282Y were shown to be either heterozygous (N = 2) or homozygous (N = 1) for HLA-A3. Patients homozygous for the C282Y mutation had higher ferritin levels and lower age at onset, but the difference was not significant. The presence of C282Y homozygosity in roughly half of the Brazilian patients with HH, together with the findings of HLA-A homozygosity in C282Y-negative subjects, suggest that other mutations in the HFE gene or in other genes involved in iron homeostasis might also be linked to HH in Brazil.

### Key words

- Hemochromatosis
- HFE mutations
- Iron overload
- HLA-A3 mutation
- C282Y mutation
- H63D mutation
- Chromosome 6

## Introduction

Hereditary hemochromatosis (HH) is an autosomal recessive disorder of iron metabolism that is very common in Caucasians of Northern European ancestry. The disorder is characterized by systemic iron overload

due to enhanced iron absorption in the small bowel (1). Progressive iron accumulation ultimately leads to organ damage and development of cirrhosis, diabetes mellitus, panhypopituitarism, cardiomyopathy, arthritis, and skin hyperpigmentation (2).

The hemochromatosis gene is found in

the major histocompatibility complex class I region on the short arm of chromosome 6, approximately 4.5 kb telomeric to the HLA-A locus. In this regard, most patients of Northern European ancestry with HH have been shown to be homozygous for HLA-A3 (3,4). In 1996, the hemochromatosis gene, now called HFE, was cloned and shown to code for an HLA class I-like protein that requires interaction with  $\beta$ 2-microglobulin for its expression on the cell surface (5,6). The HFE protein is heavily expressed in duodenal crypt cells, in association with  $\beta$ 2-microglobulin and transferrin, and has been shown to regulate the transferrin receptor-dependent iron uptake by these cells (6-9).

Two missense mutations were identified initially in the HFE gene in Caucasian patients with HH, namely a G to A transition at nucleotide 845 which leads to a substitution of cysteine for tyrosine at amino acid position 282 (C282Y) and a C to G change at nucleotide 187 that results in a substitution of histidine for aspartic acid at position 63 (H63D) (5). The C282Y mutation has been shown to disrupt the interaction between  $\beta$ 2-microglobulin and the HFE protein and to prevent its cell surface expression, while the H63D mutation is thought to alter the conformation of the HFE gene product, decreasing its affinity for ligands (6).

Most Caucasian patients with HH are homozygous for the C282Y mutation, while less than 6% of them are compound heterozygotes for C282Y and H63D (10). Indeed, the C282Y mutation is very common in populations of Northern European ancestry, with a frequency of 5 to 10%, but is very rare in other ethnic groups from Asia and Africa. In this respect, approximately one third of Italian patients with HH lack the aforementioned mutations in the HFE gene (11). It is noteworthy that these mutations are also absent in African American patients (12) as well as in patients with African iron overload (13), a hereditary syndrome also associated with increased iron

body stores (14).

The prevalence of C282Y and H63D mutations in Brazilian patients with HH remains unknown. Population analysis of these HFE mutations has shown that the allelic frequency of C282Y is 3- to 8-fold lower in Brazilians when compared to Northern European Caucasians, whereas the allelic frequency of H63D is quite similar in both population groups (11,15,16). The disease also seems to be rare in Brazil, accounting for approximately 1% of the causes of end-stage liver disease that require liver transplantation in this country (17).

The purpose of the present study was to determine the frequencies of HLA-A antigens and the prevalence of the C282Y and H63D mutations of the HFE gene in Brazilian patients with HH, as well as to compare clinical and laboratory profiles of patients with HH with and without the C282Y mutation.

## Patients and Methods

### Subjects

Fifteen unrelated male patients (median age 44 (20-72) years) with HH were studied. The diagnosis of HH was based on 1) absence of secondary causes of iron overload such as chronic hemolytic anemia, thalassemia major, sideroblastic and spur cell anemia, parenteral or dietary iron overload, alcohol abuse and chronic liver disease due to hepatitis C and non-alcoholic steatohepatitis; 2) transferrin saturation greater than 50%; 3) ferritin levels greater than 300  $\mu$ g/l, and 4) grade III or IV siderosis by Perls stain and no other evidence of other chronic liver diseases in a liver biopsy (2). The clinical, laboratory and histological features of the patients are summarized in Table 1.

### DNA extraction

Genomic DNA was extracted from pe-

ipheral blood leukocytes using the DTAB/CTAB technique (18).

### HLA typing

Determination of HLA-A antigens was performed in all patients using Dynal kits (Dynal Biotech Ltd., Bromborough, UK).

### Detection of C282Y and H63D mutations

HFE mutations were detected by PCR-RFLP analysis (19). The length of the amplified fragment of exon 4 of the HFE gene is 400 bp. The G to A transition at nucleotide 845 (amino acid 282) creates a *Sna*I cleavage site and fragments of 290 and 110 bp after endonuclease digestion. In the presence of the H63D mutation, only the undigested 208-bp fragment is observed, since the C to G transversion at nucleotide 187 (amino acid 63) disrupts a *Bcl*I cleavage site. The fourth exon of the HFE gene flanking the *Sna*I recognition site for the C282Y substitution was amplified using the following primers: 5' TGGCAAGGGTAAACAG ATCC 3' and 5' CTCAGGCACTCCTCTC AACC 3'. Amplification of the second exon of the HFE gene containing a *Bcl*I recognition site for H63D was done using the primers 5' ACATGGTTAAGGCCTGTTGC 3' and 5' GCCACATCTGGCTTGAAATT 3'. Approximately 800 and 200 ng of genomic DNA were used, respectively, for amplification of exon 2 and 4 of the HFE gene, with 0.6  $\mu$ M of each primer in a total volume of 50  $\mu$ l containing 200  $\mu$ M of each dNTP (Gibco-BRL, New York, NY, USA), 2 IU of Taq polymerase (Cenbiot, Porto Alegre, RS, Brazil) and PCR buffer containing 1.5 mM magnesium chloride. Amplification was carried out in a PTC-100 Thermal Cycler (MJ Research Inc., Watertown, MA, USA) and PCR conditions were the same for both amplifications: 96°C for 2 min, followed by 35 cycles at 96°C for 30 s, 56°C for 1 min and 72°C for 1 min. After digestion with 10 IU of *Sna*I

and *Bcl*I (Life Technologies, Bethesda, MD, USA) for 2 h at 37° and 50°C, respectively, the PCR products were visualized after electrophoresis on a 4% Nusieve agarose gel containing 50 ng ethidium bromide/ml gel by UV transillumination.

### Statistical analysis

Clinical and laboratory features of patients with and without the C282Y and H63D mutations were compared by the Fisher exact test or the Kruskal-Wallis test when appropriate. A P value <0.05 was considered significant. Data are reported in the text and tables as median and range.

### Results

The results of HLA-A and C282Y and H63D determinations in patients with HH are shown in Table 2. Eight patients (53%)

Table 1. Clinical and laboratory features of 15 Brazilian patients with hemochromatosis.

Age at onset (years)	44 [20-72]
Signs and symptoms	
Chronic liver disease	11 (73)
Diabetes	3 (20)
Impotence	3 (20)
Skin hyperpigmentation	1 (7)
Panhypopituitarism	1 (7)
Cardiac insufficiency	1 (7)
Arthritis	1 (7)
Laboratory data	
Transferrin saturation, % (normal: <45)	94 [55-100]
Ferritin, $\mu$ g/l (normal: <300)	950 [700-13,170]
ALT, IU/l (normal: $\leq$ 20)	55 [20-187]
Bilirubin, mg/dl (normal: $\leq$ 1.1)	1.7 [0.6-7.2]
Albumin, g/dl (normal: 3.5-5.0)	4.5 [2.7-4.9]
Liver biopsy	
Grade III siderosis	5 (33)
Grade IV siderosis	10 (66)
Cirrhosis	10 (66)

Signs and symptoms are reported as number of patients, with percentage in parentheses. Laboratory data are reported as medians, with range in brackets. Liver biopsy data are reported as number of patients, with percentage in parentheses.

had HLA-A3, but only two were homozygous. Eight patients (53%) were homozygous and one (7%) was heterozygous for the C282Y mutation. Only one patient carried the H63D mutation in the heterozygous state and none had compound heterozygosity. All but three C282Y homozygotes were positive for HLA-A3. However, only two were homozygous for both the HLA-A3 and C282Y mutation. Conversely, three other patients without C282Y were found to be either heterozygous (N = 2) or homozygous (N = 1) for HLA-A3.

Comparison of clinical and laboratory profiles between patients with and without homozygosity for the C282Y mutation after exclusion of one C282Y heterozygote revealed that the former patients had higher ferritin levels, as well as lower age at disease onset. However, the difference was not statistically significant (data not shown).

## Discussion

The present data demonstrate that roughly half of the Brazilian patients with HH studied here are homozygous for the C282Y mutation and that none are C282Y and H63D compound heterozygotes. The majority of

the patients who carried C282Y in the present study were homozygous or heterozygous for HLA-A3, but three patients who lacked C282Y were positive for HLA-A3, including one homozygous for HLA-A3.

These findings are different from those reported for Northern Europe, where more than 90% of the patients are C282Y homozygotes or C282Y and H63D compound heterozygotes (5,20-25), but agree with recent data reported for Italy, where approximately one third of the patients with HH showed neither C282Y or H63D mutations, nor any other mutation in the HFE gene by sequence analysis (26-28). Similarly, none of these mutations were detected in African Americans with HH or in subjects with African iron overload, another hereditary disorder of iron metabolism that is much more heterogeneous than HH (12,13). In fact, in all populations where the frequency of the C282Y mutation is negligible, the disease seems to be rare or the prevalence of the C282Y mutation in HH is lower than expected (11,29,30).

In view of the genetic heterogeneity observed in the prevalence of these mutations in patients with HH from different ethnic groups, it was not unexpected to find a lower frequency of C282Y and H63D mutations in patients with HH from Brazil, where the population is of highly admixed origin with varying percentages of Negroid, Caucasian and Amerindian ancestries (31,32). It is therefore possible that other still unknown mutations in the HFE gene or in other loci involved in iron metabolism are related to HH in Brazilian patients. In this regard, a novel locus in the long arm of chromosome 7, encoding transferrin receptor 2 (TFR2), was recently associated with non-HFE-linked HH in Italian families. Most affected siblings from these families were homozygous for a substitution of tyrosine for a stop codon at position 250 (Y250X) of the TFR2 protein (33). Nevertheless, the functional relevance of this mutation and its impact on non-

Table 2. Determination of HLA-A frequencies and C282Y and H63D mutations in Brazilian patients with hemochromatosis.

Patient	Age at onset (years)	Symptoms	C282Y	H63D	HLA
1	38	Impotence	-/-	-/-	A2 A36
2	45	Chronic liver disease	-/-	-/-	A3 0
3	49	Chronic liver disease	-/-	-/-	A3 A24
4	37	Chronic liver disease	-/-	-/-	A3 A24
5	20	Panhypopituitarism	-/-	-/-	A24 A33
6	72	Chronic liver disease	-/-	+/-	A30 A32
7	44	Chronic liver disease	+/-	-/-	A1 A30
8	40	Liver enzyme abnormalities	+/+	-/-	A1 0
9	36	Chronic liver disease	+/+	-/-	A1 A11
10	48	Chronic liver disease	+/+	-/-	A2 0
11	46	Chronic liver disease	+/+	-/-	A1 A3
12	31	Impotence	+/+	-/-	A3 0
13	38	Liver enzyme abnormalities	+/+	-/-	A3 0
14	47	Chronic liver disease	+/+	-/-	A3 A11
15	60	Chronic liver disease	+/+	-/-	A3 A23

HFE-linked HH in other countries still remains unknown.

It is also possible that other mutations in the HFE gene could be linked to HH in Brazilian patients without C282Y. In this respect, other variants, including S65C, I105T and G93R and also a splice site mutation (IVS3 + 1G → T) have been described in the HFE gene in Caucasian patients with HH, mainly in C282Y heterozygotes who lacked H63D (34-36). The S65C variant was found to be in linkage disequilibrium with HLA-A32 and the I105T and G93R mutations were linked to HLA-A3-B7 and HLA-A2-B62 haplotypes, respectively (34). Thus, sequence analysis of the entire HFE gene in the patients from this cohort would be interesting in order to find other mutations, especially in patient number 2, who lacked C282Y and H63D mutations and was homozygous for HLA-A3.

Comparison of the clinical features of patients with and without C282Y showed that C282Y-positive subjects tended to have higher ferritin levels and also lower age at disease onset, but the difference was not significant, probably due to the small number of patients studied. However, it is interesting to emphasize that a previous study has shown that iron overload in patients with HH was higher in C282Y homozygotes when compared to heterozygotes or to patients without C282Y (37).

It is also worthwhile to highlight that two

patients in this study had disease onset earlier than expected for classical HH (Table 2). One of them, who was 31 years of age at onset, had the classical HLA-A3-C282Y genotype. However, the other, who began to present symptoms of panhypopituitarism at 20 years of age, carried none of these HFE mutations. This last patient now would probably be classified as having juvenile hemochromatosis, a hereditary iron overload syndrome characterized by an earlier onset of symptoms and a higher frequency of hypogonadotropic hypogonadism and cardiac failure (38,39). This disease variant was thought to be restricted to Northern European Caucasians and was recently linked to genes in the long arm of chromosome 1 (40).

We have shown that C282Y and H63D mutations are present in about two thirds of the Brazilian patients with HH studied here. Therefore, other mutations in the HFE gene or in other genes involved in iron homeostasis might be linked to HH in Brazil. These results should be taken into account in the evaluation of diagnostic algorithms and in screening protocols for HH in this country.

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