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Research Article

Ancestral association between *HLA* and *HFE* H63D and C282Y gene mutations from northwest Colombia

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Abstract

A significant association between HFE gene mutations and the HLA-A*03-B*07 and HLA-A*29-B*44 haplotypes has been reported in the Spanish population. It has been proposed that these mutations are probably connected with Celtic and North African ancestry, respectively. We aimed to find the possible ancestral association between HLA alleles and haplotypes associated with the *HFE* gene (C282Y and H63D) mutations in 214 subjects from Antioquia, Colombia. These were 18 individuals with presumed hereditary hemochromatosis ("HH") and 196 controls. The HLA-B*07 allele was in linkage disequilibrium (LD) with C282Y, while HLA-A*23, A*29, HLA-B*44, and B*49 were in LD with H63D. Altogether, our results show that, although the H63D mutation is more common in the Antioquia population, it is not associated with any particular HLA haplotype, whereas the C282Y mutation is associated with HLA-A*03-B*07, this supporting a northern Spaniard ancestry.

Keywords: hereditary hemochromatosis, HLA class I genes, HFE gene, H63D, C282Y.

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Introduction

Hereditary hemochromatosis (HH, OMIM #235200) is an autosomal recessive disorder of iron metabolism, characterized by increased iron absorption leading to severe iron deposition and damage of different organs (Janssen and Swinkels, 2009). HH is most often caused by at least two missense mutations in the *HFE* gene: the c.C187G mutation of exon 2, which results in the substitution of histidine by aspartic acid at amino acid 63 (H63D) and the c.G845A mutation of exon 4, which results in the substitution of cysteine by tyrosine at amino acid 282 (C282Y). The *HFE* gene is located on the short arm of chromosome 6 (6p21.3) approximately 4 Mb teloremic to the histocompatibility leukocyte antigen (HLA-A) locus (Feder *et al.*, 1996) and linkage disequilibrium has been demonstrated between particular HLA-A and -B alleles and the *HFE* mutations. Indeed, C282Y has been reported to be associated with the HLA-A*3 allele and particularly with the HLA-A*3-B*7 haplotype ("the Celtic ancestral haplotype") (Simon *et al.*, 1987) in different populations (Distante *et al.*, 2004; Barton *et al.*, 2005) including the Spanish inhabitants (Pacho *et al.*, 2004). The H63D mutation has been found more frequently in Mediterranean populations associated with the HLA-A*29 allele and HLA-A*29-B*44 haplotype (Porto *et al.*, 1998; Cardoso *et al.*, 2002; Pacho *et al.*, 2004). Therefore, the HLA-A-B and the HFE haplotypes are excellent markers to study the diversity of human population and migratory processes.

We reported the presence of the C282Y mutation in eight out of 13 HH patients and one patient with the H63D mutation in Antioquia, a northwestern province of Colombia (Avila-Gomez *et al.*, 2008a). Moreover, we also found that the prevalence of H63D and C282Y in 1120 voluntary blood donors from the Antioquia region is similar to that reported from regions in central and southern Spain (Avila-Gomez *et al.*, 2008b). The Antioquia region in northwestern Colombia has approximately 5,000,000 inhabitants. This population ("paisa community") was established in the middle of the 16th and/or early 17th century by Spanish and Sephardic Jewish settlers (Carvajal-Carmona *et al.*, 2000) and remained in relative isolation until the late 19th century (Arcos-Burgos and Muenke, 2002). Based on these data, we proposed that the C282Y and H63D HFE mutations found in Antioquias settlers are probably connected with their Celtic (Soto *et al.*, 2000) and North Afri-

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can (Merryweather-Clarke *et al.*, 2000) ancestry. To test this hypothesis, this study aimed to find the possible ancestral association between HLA alleles, haplotypes with the *HFE* gene (C282 and H63D) mutations in a sample of presumed HH individuals and deceased organ donors from the Antioquia population.

Subjects and Methods

Presumed "HH" individuals and controls

In this study DNA samples from 18 unrelated individuals with presumed hereditary hemochromatosis ("HH"*)* were studied; 13 of these subjects were *HFE* genotyped within a previous survey (Avila-Gomez *et al.*, 2008a) and five new individuals were recruited at Hospital San Vicente de Paul (Medellin, Colombia). A written informed consent was obtained from all subjects. The control group was composed of 196 deceased donors for organ transplants in Medellin (Colombia) between 2000 and 2009, whose DNA samples were stored at Laboratory de Inmunología de Transplantes del Grupo de Inmunología e Inmunogenética (GICIG) de la Universidad de Antioquia. These donors were HLA-A,-B,-DRB1 typed by PCR-SSP after a written informed consent was granted by the donors' relatives, according to the Colombian legislation for organ and tissue donation. For the HFE typing, the control samples were selected by proportional stratified random sampling in order to have a number of donors per year that was proportional to the total number of donors studied each year.

Detection of H63D and C282Y mutations by PCR-RFLP

The H63D (nucleotide change c.187 C > G) and C282Y (c.845 G > A) mutations were tested by polymerase chain reaction (PCR) and restriction fragment length polymorphism (RFLP) analysis, using the forward and reverse primers and PCR conditions described by Merryweather-Clarke *et al.* (1998) and Takeuchi *et al.* (1997), respectively. For H63D, the amplified fragments were digested with *Mbo*-I and the 294 bp PCR product of H63D (exon 2) region showed three fragments of 140, 99 and 55 bp in wild type DNA, and only two fragments of 239 and 55 bp in mutant DNA. The PCR product of the 367 bp C282Y (exon 4) region was digested with *Rsa*-I and generated fragments of 278 and 89 bp in normal DNA and 249, 89 and 29 bp in mutant DNA. PCR digests were analysed on 2.5% agarose gels.

HLA - AB typing by PCR-SSP

DNA samples were extracted by salting out from buffy coats obtained from spleen or EDTA-anti-coagulated blood. The DNA concentration of each sample was adjusted to 150 ng/μL and typing of HLA Class I (A,B) and HLA Class II (DRB) was performed by PCR using the HLA-A+B+DRB Typing Tray Kit produced by the Collaborative Transplant Study (CTS, University of Heidelberg, Heidelberg, Germany). The PCR conditions used were: initial denaturation at 94 °C for 2 min, followed by 10 cycles of denaturation at 94 °C for 15 s and annealing extension at 65 °C for 1 min. This was followed by 20 cycles of denaturation at 94 °C for 15 s, annealing at 61 °C for 50 s, and a final extension at 72 °C for 30 s. The amplicons were detected by electrophoresis in 2% agarose gels stained with ethidium bromide. The allelic assignment was performed by amplification pattern analysis using the classification tables provided by the manufacturer.

Statistical analysis

Descriptive statistics was used to show the baseline characteristics of the groups studied. Allele and genotype frequencies were estimated using a mere gene counting procedure, and haplotype frequencies were estimated using the ELB (Excoffier-Laval-Balding) algorithm for multilocus genotypic data when the gametic phase is unknown. Linkage disequilibrium (LD) between the alleles was estimated by D and D' values; the significance of the association was tested using an extension of Fisher's exact probability test on contingency tables. To test for departures from the assumption of Hardy-Weinberg equilibrium (HWE), a test analogous to Fisher's exact test was used on a contingency table of observed *vs.* predicted genotype frequencies using a modified version of the Markov-Chain Random Walk algorithm. Statistical analyses were carried out using Arlequin version 3.0 (Switzerland) and Genepop on the web software.

Results

Demographical characteristics of control and presumed "HH" individuals

Of the 18 "HH" subjects screened for *HFE* mutations, 13 (72.2%) were men and their median age was 54 years (interquartile range 44-64). The control group included 197 deceased organ donors; among them 150 (76.1%) were men. Their median age was 26 years (interquartile range 20-41 years).

Determination of HLA-A, B frequencies according to HFE mutations

To determine whether HLA Class I alleles were associated with HFE mutations in the studied population, the "HH" individuals and controls were combined in one group and haplotypes were estimated in three or four loci. Thirty-seven HLA-A*-H63D-C282Y haplotypes were estimated, 18 of which presented a frequency above 1% and represented 92.6% of the total frequencies observed. The most frequent HLA-A* alleles associated with the H63D mutation were A*02, A*23, and A*29, while the HLA*03 allele was the most frequent with the C282Y mutation (Table 1). For the HLA-B*, H63D, and C282Y loci, 54

Table 1 - Haplotype frequencies of HLA-A*-H63D-C282Y in unrelated individuals.

HLA-A*-H63D-C282Y 2n = 428	Frequencies (%)
02, C, G	82 (19.2)
24, C, G	75 (17.5)
30, C G	22 (5.1)
03, C, G	21 (4.9)
23, C G	21 (4.9)
68, C, G	20 (4.7)
31, C, G	18 (4.2)
02, **G**, **G**	**17 (4.0)**
01, C, G	16 (3.7)
33, C, G	16 (3.7)
26, C, G	16 (3.7)
11, C, G	15 (3.5)
23, **G**, **G**	**13 (3.0)**
29, **G**, **G**	**11 (2.6)**
32, C, G	10 (2.3)
03, G, G	9 (2.1)
03, **C**, **A**	**8 (1.9)**
29, C, G	7 (1.6)

Table 2 - Haplotype frequencies of HLA-B*-H63D-C282Y in unrelated individuals.

HLA-B*-H63D-C282Y 2n = 428	Frequencies (%)
35, C, G	78 (18.2)
44, C, G	28 (6.5)
39, C, G	23 (5.4)
18, C, G	23 (5.4)
51, C, G	22 (5.1)
44, **G**, **G**	**19 (4.4)**
65, C, G	16 (3.7)
61, C, G	15 (3.5)
07, C, G	14 (3.3)
58, C, G	11 (2.6)
35, **G**, **G**	**11 (2.6)**
38, C, G	11 (2.6)
50, C, G	10 (2.3)
60, C, G	9 (2.1)
49, **G**, **G**	**9 (2.1)**
08, C, G	8 (1.9)
57, C, G	7 (1.6)
07, **C**, **A**	**7 (1.6)**
49, C, G	6 (1.4)
63, C, G	6 (1.4)
62, C, G	6 (1.4)
40, C, G	5 (1.2)
53, C, G	5 (1.2)
41, C, G	5 (1.2)
52, C, G	5 (1.2)
72, C, G	5 (1.2)
27, C, G	5 (1.2)
18, G, G	5 (1.2)

HLA-B*-H63D-C282Y haplotypes were estimated, 28 of which presented a frequency above 1% and represented 87.5% of the total frequencies observed (Table 2). The most frequent alleles with the H63D mutation were B*44, B*35, and B*49, while the most frequent B allele associated with the C282Y mutation was HLA-B*07. Additionally, 173 HLA-A*-B*-H63D-C282Y haplotypes were estimated, 21 of which presented a frequency above 1% and represented 45.4% of the total frequencies observed (Table 3). The most frequent HLA haplotypes with mutation in the H63D locus were HLA-A*29-B*44, HLA-A*03-B*35, HLA-A*02-B*44, and HLA-A*23-B*49. The most frequent haplotype with the C282Y mutation was HLA-A*03-B*07. The HLA-A*29, B*44, and B*49 alleles were in linkage disequilibrium with the H63D mutation ($D = 0.50$; $D = 0.24$; $D = 0.52$, respectively, $p < 0.0001$). In the same manner, HLA-A*03 ($D = 0.35$) and HLA-B*07 ($D = 0.38$) were in linkage disequilibrium with the C282Y mutation.

Discussion

The immigration of Spaniards to the Americas was massive in the late 16th and early 20th century. This population movement is in accordance with the "demic-diffusion" model (Carvajal-Carmona *et al.*, 2000; Bedoya *et al.*, 2006). Hence, the HLA allele and haplotype and the prevalence of the *HFE* mutations in the Americas, particularly in Colombia, would be expected to be directly influenced by this migration model. This is the first ancestral association study between HLA haplotypes and *HFE* gene mutations conducted in Colombia, and particularly in the northwestern province of Antioquia. Our results confirm the association between the HLA-A*03-B*07 haplotype and the C282Y mutation reported in many populations (Barton andActon, 2002; Distante *et al.*, 2004; Pacho *et al.*, 2004; Barton *et al.*, 2005). We also found that the HLA-A*03-B*35 haplotype associated with the H63D mutation is increased in the studied population. Interestingly, the HLA-A*03-B*35 has been linked to hemochromatosis in north-eastern regions of Italy (Panajotopoulos *et al.*, 1989; De Menis *et al.*, 1990), but not so in Spain, where there is a significant association between the HLA-A*03-B*62 and HLA-A*03-B*44 haplotypes and the C282Y mutation (Pacho *et al.*, 2004). Since HLA-B*35 is one of the commonest allele (17.8%) in paisa population (Rodríguez *et al.*, 2007), HLA-A*03-B*35 may represent a distinctive haplotype marker of Antioquia population

Table 3 - Haplotype frequencies of HLA-A*-B*-H63D-C282Y in unrelated individuals.

HLA-A*-B*-H63D-C282Y 2n = 428	Frequencies (%)
24, 35, C, G	45 (10.5)
02, 39, C, G	16 (3.7)
02, 35, C, G	10 (2.3)
02, 44, C, G	10 (2.3)
03, 07, C, G	9 (2.1)
33, 65, C, G	9 (2.1)
02, 18, C, G	9 (2.1)
29, **44**, **G**, **G**	**8 (1.9)**
02, 51, C, G	8 (1.9)
26, 38, C, G	8 (1.9)
03, **35**, **G**, **G**	**7 (1.6)**
03, 35, C, G	6 (1.4)
68, 18, C, G	6 (1.4)
02, **44**, **G**, **G**	**6 (1.4)**
23, **49**, **G**, **G**	**6 (1.4)**
03, **07**, **C**, **A**	**6 (1.4)**
24, 61, C, G	5 (1.2)
02, 50, C, G	5 (1.2)
24, 18, C, G	5 (1.2)
11, 35, C, G	5 (1.2)
31, 61, C G	5 (1.2)

(21.5%) frequency (Rodríguez *et al.*, 2007). Recently, Olsson *et al.* (2010) suggested that HLA-A*03-B14 and HLA-A*03-B*07 might be the ancestral haplotypes of the C282Y mutation in a Scandinavian family with major iron overload. Taken together, our data support that the C282Y mutation found in Antioquia might be connected with the Celtic ancestral A*03-B*07 haplotype (Soto *et al.*, 2000).

We have previously proposed that whilst the C282Y HFE mutation found in Antioquia is probably connected with Celtic ancestry (Pacho *et al.*, 2004), the H63D mutation might be originally connected to a North African population (Merryweather-Clarke *et al.*, 2000). It is generally well accepted that the C282Y mutation arose from a Celtic ancestry (Lucotte and Dieterlen, 2003; Milman and Pedersen, 2003) some 60 generations ago, before 4000 B.C. (Distante *et al.*, 2004). Consequently, it has been postulated that the gradient distribution of frequencies in hemochromatosis is similar to the Celtic or Viking migration streams in Europe. In Spain, the Celtic invasion initiated in the 9th Century B.C. and lasted until 500 B.C., resulting in a brand new populace known as Celtiberian people (Celts plus native Iberians), which in turn spread from the north to the middle of the Iberian Peninsula (Lorrio and Ruiz-Zapatero, 2005). Accordingly, the northern regions of Spain, such as Galicia, Asturias, Cantabria, and Basque Country present high C282Y allele frequencies (3-5%) similar to the frequencies of Wales (UK) or Toulouse (France) (Merryweather-Clarke *et al.*, 1998). These results have been interpreted as a Celtic legacy by the well-matched high C282Y frequency, historical records and/or geographical isolation (Soto *et al.*, 2000). Thus, the strong association between the HLA-A*03-B*07 haplotype and the C282Y mutation found in Antioquia suggests that this mutation is originally connected with Celtic ancestry through the Spaniards' settlement.

Because the origin of the H63D mutation is less certain and because the highest H63D frequency was detected in Spain, Aguilar-Martinez *et al.* (1999) hypothesized that the H63D mutation emerged in the Iberian Peninsula. Alternatively, the H63D could have arisen in the Middle-East or in Northern Africa. Indeed, H63D is found in countries bordering the Mediterranean, the Middle East, and in the Indian subcontinent (Merryweather-Clarke *et al.*, 2000). Furthermore, the allele frequency of C282Y is extremely low (0.9-0.09%) (Sassi *et al.*, 2004; Ezzikouri *et al.*, 2008) or inexistent (Jeffery *et al.*, 1999; Roth *et al.*, 1997; Gunel-Ozcan *et al.*, 2006; Settin *et al.*, 2006) in African countries and Turkey. These data clearly suggest that the C282Y mutation is restricted to Europeans, but the H63D is not. These observations suggest that the H63D mutation may have arisen more than once in geographically distant regions (Rochette *et al.*, 1999) and that this mutation may be older than the C282Y mutation (Distante *et al.*, 2004). Additionally, the H63D allele frequencies found in Spain are similar to the allele frequency reported from the Sephardic Jewish (Matas *et al.*, 2006), Morroquian and Algerian (Aguilar-Martinez *et al.*, 2001; Ezzikouri *et al.*, 2008), Tunisian (Sassi *et al.*, 2004) and Turkish (Simsek *et al.*, 2004) populations. Because Jews traditionally seldom intermarry and no historical records of population movements (invasions or massive migration) exist that could associate Turkish with Spanish people, the conquest of the former Roman province of Hispania by the Moors between 711-1492 A.D. certainly marked the history, culture and most probably the genetic makeup of the Spanish population (Lucotte *et al.*, 2001; Gérard *et al.*, 2006).

Although at present no data is available on the association between HLA haplotype and HFE mutations in African countries, Martinez-Laso *et al.* (1995) reported that HLA haplotypes in Basques and Spaniards are closer to paleo-North African populations than to other Europeans. Moreover, the fact that Basques, Spaniards and Algerians shared common HLA haplotypes (*e.g.*, A*33-B*14-DR1) and even specific haplotypes (*e.g.*, Basques and Algerians A*11-B*27-DR1 and A*02-B*35-DR11), suggests a relative high admixture with North-Africans (Arnaiz-Villena *et al.*, 1997). Altogether, our results show that, although the H63D mutation is more common in the Antioquia population, it is not associated with any particular HLA haplotype, whereas the C282Y mutation is associated with

HLA-A*03, B*07 and supports its presence through the northern Spaniard ancestry.

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