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SHORT REPORT

HFE gene mutations analysis in Basque hereditary haemochromatosis patients and controls

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C282Y/C282Y genotype is the prevalent genotype in Hereditary Haemochromatosis (HH), however, other genotypes have been associated with the disease. The objective of our study was to analyse the frequency of the three main mutations of HFE gene in HH patients and controls from the Basque population with differential genetic characteristics. Thirty five HH patients and 116 controls were screened for C282Y, H63D and S65C mutations using a PCR–RFLP technique. The association of HLA-A and-B alleles and HFE mutations was also studied in Basque controls. The frequency of C282Y homozygotes in the group of patients was only 57%. The rest of the patients presented heterogeneous genotypes, including compound heterozygotes: 11% of them were C282Y/H63D; and 2.85% were H63D/S65C. H63D or S65C heterozygotes had a frequency of 11% and 2.85 respectively and 5.71% patients lacked any mutation The high frequency of H63D in the healthy Basque population is confirmed in this study. A considerable incidence of S65C is observed either in controls and in HH (3%) or in iron overloaded patients. The peculiar genetic characteristics of Basques could explain the heterogeneity of genotypes in HH patients of this group. Further studies should be carried out to confirm these findings although the implication of other genetic or external factors in the development of HH is suggested.
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Introduction

Hereditary haemochromatosis (HH) is a very common autosomal recessive disease with especially high prevalence in northern European populations. The disease affects the iron metabolism, the excess of body iron accumulates in the liver and other organs resulting in liver damage and other clinical disorders eg cirrhosis and hepatocellular carcinoma which can be prevented by early diagnosis and treatment with flebotomy.

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In 1976, Simon *et al* discovered that the genetic predisposition cosegregated with HLA-A3 allele indicating its closing link to HLA.[1] In 1996, the haemochromatosis gene *HFE* was cloned.[2] The *HFE* gene product is a major histocompatibility complex (MHC) class I-like molecule that binds $\beta 2$ microglobulin. It appears to complex with the transferrin receptor lowering its affinity for transferrin. Two causative mutations have been described in *HFE* gene. The main one results in a substitution of a tyrosine for a cysteine at 282 position (C282Y). The second results in an aspartic acid for a histidine at position 63 of the protein (H63D). Several studies have demonstrated that C282Y mutation disrupts the interaction between HFE molecule and $\beta 2$ microglobulin and its cell surface localisation whereas H63D change do not avoid the protein to be expressed but could simply impair its interaction with the transferrin receptor.[3]

C282Y is the most prevalent mutation in HH patients. Between 60 and 100% of patients are homozygous for this mutation with a decreasing gradient from north to south.[4] Among the patients that are not homozygous for C282Y there are: compound heterocygotes; H63D homocygotes; C282Y heterozygotes and H63D heterozygotes. Between 4–20% of HH patients have neither of these mutations. The *HFE* gene of many of these patients have been sequenced and most times no other mutations have been found. However other mutations or variants have been described in *HFE* gene either in iron overload conditions or in healthy individuals. Among them, S65C, an adenine to thymine transversion (193 A to T) which results in a conservative substitution have been found in several series of patients and may be implicated in the development of a milder form of HH.[5]

Frequency of the main mutations in HH: H63D and C282Y have been studied in a complete study with populations worldwide.[6] This study showed that whereas C282Y is restricted to European populations H63D has a more general distribution and its highest frequency is localised in the Basque population. The Basque population is considered to have genetic peculiarities as reported in many studies.[7] Although its origin is controversial, it is generally accepted that Basques have mixed with invading populations to a lesser extent.[8] Concerning the HLA system, where an *HFE* gene is located, substancial differences have been reported in HLA haplotypes and HLA allele frequencies when compared with other close populations.[9]

Our objectives are first, to study the frequency of the three main mutations in *HFE* gene: C282Y, H63D and S65C in a group of HH patients and controls from Basque origin and second study the interactions between HLA alleles and HFE mutations in this population.

Patients and methods

The group of patients consisted of 35 unrelated Basque individuals with primary haemochromatosis (25 men and 10 women) with a median age of 49 years (range: 26–71). They presented a median ferritin value of 1121 μg/l (range: 350–3255); a median transferrin saturation percentage of 70 (range: 40–100%); and a median serum iron of 175 mg/dl (range:110–248). Six out of 35 patients had familial history of disease (first degree relatives). Patients with haematological disorders and other causes of iron overload were excluded from the study. The diagnosis of HH was based on clinical history and on having at least two of the following criteria: (1) increased tranferrin saturation, repeatedly greater than 50%; (2) histological iron of grade III to IV and (3) hepatic iron index equal or greater than 1.9.[10] In 12 cases, liver biopsy could not be done and the diagnosis of haemochromatosis was confirmed by the mobilisation of a total amount of iron greater than 4 g in phlebotomy.[11] All healthy controls were recruited from the provincial blood donor bank. The Basque origin of this group was validated by their characteristic Basque surnames in the last three generations.

Genomic DNA was isolated from whole EDTA blood with QIAGEN (Germany) silicagel columns following the manufacturer's protocol. H63D, C282Y and S65C mutations were studied by PCR–RFLP technique with oligonucleotides described elsewere.[6] Amplifications were performed in a Perkin Elmer thermocycler in standard conditions; annealing temperatures: 58°C for H63D and S65C and 61°C for C282Y. Then, PCR products were digested with *Sau*3AI (H63D)and *Rsa*I (C282Y) and *Hinf*I (S65C) and the result fragments were separated by electrophoresis in a 2.5% TBE agarose gel and detected by ethidium bromide staining.

Genotype and allele frequencies, together with 95% of confidence limits, were estimated for the HFE mutations in both groups of patients (n=35) and controls (n=116) (Tables 1 and 2). HLA class I: -A, -B antigens were identifed by standard microlymphocytotoxicity technique and allele frequencies were calculated[9] in a group of 84 controls. The existence of association or linkage disequilibrium between HFE mutations and HLA-A and B alleles was tested in the group of controls. The level of significance of those associations was calculated by 2×2 comparison tables and P values were calculated using Yates corrected Chi^2 and Fisher's tests. P values were corrected according to the number of alleles compared.[12]

Results

Genotype frequencies were analysed in patients and control group and they are shown in Table 1. Only 20 out of 35 patients of this group (57%) were found to be homozygous for C282Y mutation. The rest of them presented heterogeneous genotypes: Four patients (11%) were compound heterozygotes (H63D/C282Y); other four (11%) were H63D heterozygotes and three (8%) were C282Y carriers. S65C was found in two patients: one was a H63D/S65C compound heterozygote and the other one was a heterozygote for S65C mutation only. Two out of 35 (5.71%) lacked any of the three mutations studied.

S65C mutation, poorly studied in other populations, was also represented in control group with a 3% of allelic frequency (Table 2). This mutation was also found in eight other patients studied for HFE mutations due to different grades of iron overload based on biochemical and clinical grounds but without a liver biopsy or other criteria to diagnose HH. Two of them were S65C/C282Y herterozygotes; two were S65C/H63D and the other four were S65C heterozygotes.

H63D mutation presented a higher allelic frequency in Basque patients (13%) when compared with Spanish[13] and other Caucasoid series of patients from southern Europe but this increase is not significant when data were corrected for the number of C282Y negative chromosomes :39% H63D in Basque HH and 38% of average frequency in European patients series.[4] This mutation is highly represented in our

Table 1 Distribution of genotype frequencies in Basque patients and controls

			Controls		*Patients*	
H63D	*C282Y*	*S65C*	n	*%*	n	*%*
−/−	−/−	−/−	43	37.07	2	5.71
+/−	−/−	−/−	45	38.79	4	11.43
−/−	−/+	−/−	8	6.90	3	8.57
−/−	−/−	−/+	5	4.31	1	2.86
+/+	−/−	−/−	9	7.76	0	–
−/−	+/+	−/−	0	–	20	57.14
−/−	−/−	+/+	0	–	0	0
+/−	+/−	−/−	4	3.45	4	11.43
+/−	−/−	+/−	2	1.72	1	2.86
−/−	+/−	+/−	0	–	0	–
	Total		116		35	

Table 2 Mutations in the HFE gene: allele frequencies (% 95% confidence limits)

	Controls n=*116*	*Patients* n=*35*
H63D	29.31 (22.6–43)	12.85 (6.06–23.01)
C282Y	5.17 (2.8–8.7)	67.14 (54.88–77.91)
S65C	3.01 (1.3–6.2)	2.85 (0.35–9.94)

Basque control group (29.31%). H63D seemed to be associated to HLA-A29 and B44 alelles in the group of Basque controls (P=0.03 and P=0.05 respectively) although the level of significance is lost when P values are corrected according to recommendations for multiple comparisons statistics in HLA studies.[12] HLA-A29-B44 is a very frequent haplotype in Basques with a high linkage disequilibrium value.[9]

From the clinical point of view, there were not differences between the expression of the disease between HH individuals carrying an homozygous mutation and others not carrying characteristic HFE mutations.

Discussion

We analysed HFE mutations in a group of 35 HH patients and 116 healthy controls from the Basque population. A noteworthy point from this work was the low frequency of C282Y homozygotes in the group of patients (57.1%). This result differs from those relating to populations from northern Europe[4] and also from Spanish series[13,14] in which the percentage of C282Y homozygotes reached 80–90%. The HH patients, non homocygous for C282Y presented a great heterogenety of genotypes and a 6% of patients lacking any mutation (Table 1). Given the high number of normal *HFE* genes in our group of HH, we compared HFE haplotypes in these patients diagnosed without biopsy (12/35) with those having a classical diagnostic based on liver biopsy: 10 out of 12 were C282Y homozygotes, thus confirming a correct diagnosis of the disease.

In this study, H63D mutation had an allele frequency of 39% in patients and 31% in controls when data were corrected for the number of C282Y negative chromosomes. H63D frequency in patients is similar to the 38% average frequency in European patients series but it is only slightly increased in our patients *versus* controls due to the high frequency of H63D in Basques.[4] This data , already reported, is confirmed in our study with a larger group (n=116) of controls. We found a 3% allelic frequency for S65C mutation both in HH patients and controls in this study. Although these data argue against a significant pathogenic role of this mutation for HH, we found a relative incidence of S65C mutation in patients with moderate or severe iron overload. This group of patients should be systematically studied in the future in order to elucidate its real implication in hepatic iron overload. Several studies found S65C mutation in compound heterocygote states: S65C/C282Y and H63D/C282Y in patients with a mild form of HH.[5,1516]

Although the group of patients studied is small (n=35), the great heterogeneity of genotypes, different from the classical C282Y/C282Y, found in this series could reflect the heterogeneity of HH in Basque population with genetic singularities already described for other genes.[17] Our data, have similarities with the results of HH in Italy[18] and in the south of France[19] with a low frequency of C282Y homocygotes. These results and ours suggest the existence of other genetic factors possibly implicated in determining severity in iron overload and development of HH in heterozygotes or non mutated patients depending on the genetic background of the population studied.

Furthermore, recent studies about the allele expression of mutated *HFE* genotypes suggest a potential role of H63D mutated protein in the increase of iron overload in H63D heterozygotes especially in compound heterozygotes.[20] These authors postulate that in the presence of other acquired factors like heavy alcohol intake, chronic hepatitis B or C virus infectious, this mutation could enhance iron absorption and finally the development of HH.

HLA system in proximity to HFE gene had been related to HH long before the description of HFE mutations by Feder *et*

al.[2] Many studies have confirmed the existence of a long (4 Mb) ancestral haplotype (HLA-A3, B7) associated with C282Y mutation with a probable Celtic origin. Concerning H63D mutation, Feder *et al* , reported that this mutation did not present linkage disequilibrium beyond 700 Kb centromeric of HFE gene.[2] Other studies found H63D mutation mainly associated with a characteristic HFE caucasian haplotype defined by intragenic polymorphisms and tentatively assigned with an 'hispanic or mediterranean' origin.[21] On the other hand, H63D was recently found associated to HLA-A29 haplotype in a group of patients with non classical Haemochromatosis of Portuguese (Iberian) origin[22] and our results also suggest a link of H63D and HLA-A29B44 haplotype. A more exhaustive analysis of these HFE-H63D mutated haplotypes in our population could reveal a link between HLA-A29B44 alleles and the defined HFE-H63D 'hispanic' haplotype due to genetic recombinations in the past.

In conclusion, the peculiar genetic characteristics of the Basques could explain the heterogeneity of HH genotypes found in this study. Then, as others have also postulated, both the presence of other genetic or external factors could explain the appearance of a severe iron overload and HH in some of the H63D heterozygotes and no mutated genotypes. Further analysis of new genes, ie the recently described mutation in gene encoding ferroportin (SLC11A3) , associated with dominant haemochromatosis,[23] would be an interesting direction for future research.

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