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ARTICLE

Comparative study of the two more frequent *HFE* mutations (C282Y and H63D): significant different allelic frequencies between the North and South of Portugal

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An earlier study of reference values of iron parameters in Portugal showed significant differences between populations from northern and southern villages. This study addresses the question of the geographical distribution in Portugal of the two main mutations (C282Y and H63D) of the hereditary hemochromatosis gene, *HFE*. For that purpose, a stratified sample of 640 anonymous dried blood spot samples was randomly selected from the major regions of Portugal: North, Center, Lisbon and the Tagus Valley, Alentejo and Algarve. Differences in the geographical distribution of these two mutations were observed thus confirming the presumed differences between the age of the two mutations which is compatible with the postulated Celtic/Nordic origin of the C282Y mutation. The finding of a significantly higher allelic frequency of the C282Y mutation in the North (0.058) than in the South (0.009) could also point to an effect of differential selective forces acting in the different geographical areas of the country. Data on archaeological, ethnographic and linguistic records and on the North/South distribution of Portuguese cattle breeds of European or African origin have also been reported. In addition to their interest for population genetics, the results represent a reminder of the need to take into account regional differences in the design of strategies for population screening of hereditary hemochromatosis. *European Journal of Human Genetics* (2001) **9**, 843–848.

Keywords: *HFE*; hemochromatosis; C282Y; H63D; Iberian peninsula; population genetics

Introduction

Hereditary hemochromatosis (HHC) is one of the most common hereditary metabolic diseases in Caucasians. It is an autosomic recessive disorder of iron metabolism characterised by increased iron absorption that leads to iron overload of parenchymal cells in several organs.[1–3] Clinical consequences of iron accumulation include cirrhosis of the liver, hepatocellular carcinoma, diabetes, heart failure, arthritis and hypogonadism.

Feder *et al*[4] reported a candidate gene for HHC on chromosome 6 (6p22.1) as a non classical MHC-class I gene denominated *HFE*, in which two mutations were identified: C282Y and H63D. The C282Y mutation predicts substitution of the cysteine residue at codon 282 by a tyrosine in the alpha3 domain of the molecule[4] and prevents the interaction with beta2-microglobulin and consequently disrupts the HFE structure and function.[5,6] The C282Y mutation in homozygosity is responsible for the majority of HHC cases (for

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review see Porto and De Sousa[7]). The H63D mutation results in a substitution in the aminoacid 63 in the alpha1 domain changing a histidine to an aspartate.[4] The role of the H63D mutation in the pathophysiology of the disease is still unclear.

The frequency of the *HFE* mutations in control subjects has been reported in several European populations and in populations of European descent. The highest allelic frequencies for the C282Y mutation were found in the Northern European countries, namely in Ireland (an average of 10%),[8,9] with a lower frequency (1%) observed in countries from southern Europe, namely Italy.[10] The H63D mutation was found in highest frequencies in the Iberian Peninsula (above 20%).[11,12] In non-Caucasian populations *HFE* mutations were either absent or found in lower frequencies.[13,14]

The present work was motivated by a previous study of reference values of iron parameters in Portugal done before the discovery of the *HFE* gene.[15] That study reported regional differences in iron parameters between Northern and Southern villages in Portugal. These differences were observed both in serum ferritin levels in females and in serum iron levels in males from the two regions, with the highest values reported in subjects from the North. These differences were also reflected on the prevalence of iron overload and iron deficiency in the two regions. Iron deficiency was found significantly more frequently in the South, in contrast with iron overload that was found more commonly in the North.[15]

The aim of the present work was to estimate the frequency of the C282Y and H63D *HFE* mutations in Portugal, comparing its distribution through different regions.

Material and methods

The National Institute of Statistics (INE) divides Portugal in five major regions: North, Center, Lisbon and the Tagus Valley, Alentejo and Algarve (see Figure 1). Lisbon and the Tagus Valley, being an immigration region from other regions in the country reflects, to a large extent, the whole population in the country.

Sample selection

In order to compare the prevalence of the C282Y and H63D *HFE* mutations among those five regions, an anonymous random stratified sample was used. The Portuguese Neonatal Screening Program covers 95% of all newborns in Portugal. A single laboratory is responsible for the screening at the Medical Genetics Institute. The information of each card is kept in a database and the only accessed information regarding the subjects is the record number and the geographical location, constituting the sampling base.

From this base, a stratified random sample of 640 anonymous dried blood spot samples was selected, through a random number generator, from the five regions of Portugal. The selection of the sample size was based on the previous estimated allele frequency of the C282Y mutation in a control population from Northern Portugal.[12] The sample size, taking into consideration the cost and time required for the analysis, was calculated in order to assure a global error below 1% for a 95% confidence interval.

The distribution of the 640 genotyped dried blood spot samples according to the five Portuguese regions was as follows: 129 from the North, 130 from the Center, 133 from Lisbon and the Tagus Valley, 132 from the Alentejo and 116 from the Algarve.

DNA purification

The DNA purification of the dried blood samples was done using the InstaGene™ Dry Blood Kit (Bio-Rad Laboratories), according to the manufacturer's recommendations. This procedure consists in the selective extraction of amplification reaction inhibitors present in the blood without removing any of the filter-bound DNA. The extracted dry blood spot is added directly to the amplification reaction mixture prior to thermocycling.

HFE genotyping

The *HFE* genotyping was done using two commercial kits (Haemochromatosis Gene Mutation Assay I and II, Vienna-Lab, Vienna, Austria). Briefly, exon 4 (for C282Y) or exon 2 (for H63D) sequences of the *HFEgene* were amplified *in vitro* and terminally labelled with fluorescein as a reporter molecule. The amplification products were alkali-denatured, and 25 μl aliquots were selectively hybridised to allele-specific (wild type or mutant) oligonucleotide probes immobilised in two separate cavities of a microwell plate. After hybridisation and stringent washes at 37°C, bound sequences were detected using a horseradish peroxidase-labelled anti-fluorescein antibody and colour reaction with tetramethylbenzidine. The methodology, as well as its validation on samples of known genotype (RFLP-typed) and application for typing were presented elsewhere.[16–18]

Statistical analysis

The differences among the allelic frequencies in the different regions were tested by contingency table analysis (Chi-square Test). Given that the proportions were close to zero, the confidence limits for the proportions were calculated using a relationship between the F distribution and the binomial distribution. The multiple comparisons for the proportions were based on a procedure analogous to the Tukey test, based on the angular transformation of each proportion.[19]

Results

The results obtained in terms of *HFE* genotypes and the corresponding allelic frequencies of the two mutations by region are summarised in Table 1 and illustrated in Figure 1. The lower and upper 95% confidence limits for the allelic frequencies in the five regions are also presented in Table 1.

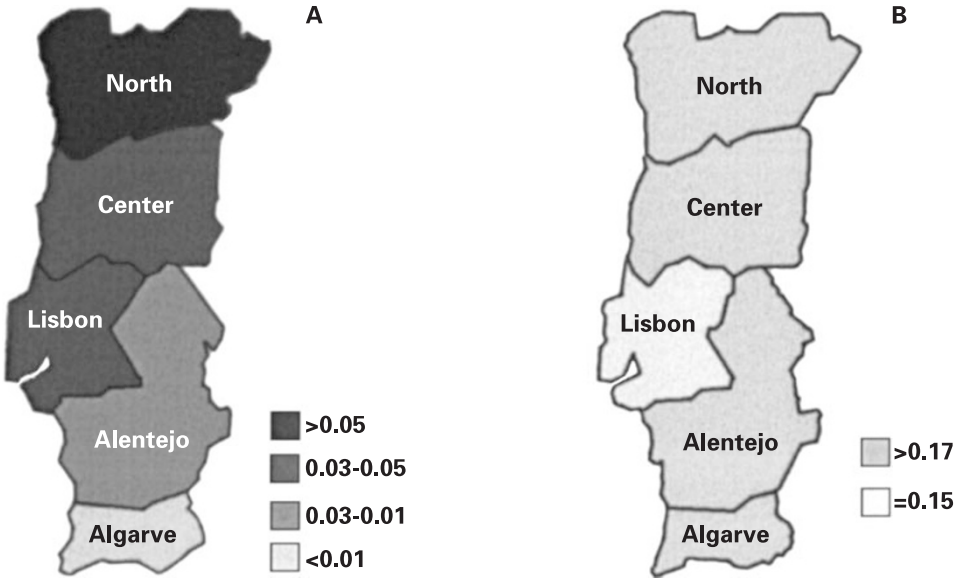

Figure 1 Maps representing the geographical distribution of the allelic frequencies of the C282Y (**A**) and H63D (**B**) *HFE* mutations in the five Portuguese regions.

Table 1 Number of subjects genotyped for the C282Y and H63D *HFE* mutations, the correspondent allelic frequencies and 95% confidence limits for the allelic frequencies in the five regions of Portugal. (*n*=total number of subjects genotyped)

HFE genotype	*North* (n=129)	*Center* (n=130)	*Lisbon and Tagus Valley* (n=133)	*Alentejo* (n=132)	*Algarve* (n=116)
C282Y/C282Y	1	1	0	0	0
C282Y/wt	10	5	7	5	0
C282Y/H63D	3	5	1	1	2
H63D/wt	31	35	27	37	23
H63D/H63D	8	6	6	5	7
wt/wt	76	78	92	84	84
C282Y allelic frequency	0.058	0.046	0.030	0.023	0.009
95% confidence interval	[0.033; 0.095]	[0.024; 0.079]	[0.013; 0.058]	[0.008; 0.049]	[0.001; 0.031]]
H63D allelic frequency	0.194	0.200	0.150	0.182	0.168
95% confidence interval	[0.151; 0.247]	[0.152; 0.250]	[0.112; 0.200]	[0.140; 0.233]	[0.126; 0.221]

C282Y *HFE* mutation

The overall distribution of the C282Y mutation includes two homozygous and 39 heterozygous samples (12 of the 39 are compound heterozygous for both mutations) (Table 1).

The allelic frequency of the C282Y mutation decreases from the North to the South. In the North, an allelic frequency of 0.058 ([0.033, 0.095] 95% CI) was observed contrasting with the allelic frequency of 0.009 ([0.001, 0.031] 95% CI) observed in the Algarve (Figure 1a, Table 1). The frequency of the C282Y mutation is not independent of the regions tested (χ^2=11.57, $P<0.05$). When the frequency of the C282Y allele was compared between the different regions, applying the Tukey test (Table 2), a significant difference was observed between the North and the Algarve (q=4.711, $P<0.05$) and between the Center and the Algarve (q=3.873, $P<0.05$). In spite of a decreasing allele frequency of the C282Y from the North to the South, no other statistically significant differences were seen with the present sample size (Table 2).

H63D *HFE* mutation

In terms of global genotype frequencies, 32 homozygous and 165 heterozygous samples (12 of the 165 are compound heterozygous for both mutations) for the H63D mutation were observed. The allelic frequency of the H63D is independent of the region tested (χ^2=2.59, $P>0.05$), ranging from 0.19 ([0.151, 0.247] 95% CI) in the North to 0.17 ([0.126, 0.221] 95% CI) in the Algarve (Figure 1b, Table 1).

In summary, the regional differences seen in the allelic frequency of the C282Y mutation between the North and the South contrasted remarkably with the homogeneous dis-

Table 2 Multiple comparisons of the C282Y allelic frequencies between the five different Portuguese regions (Tukey test). The significant differences are highlighted in bold in the table. No other significant differences between regions were found

Region 1	*Region 2*	*Allelic Diff.*	*Transf. Diff.*	*SE*	*q*
North	Center	0.011986	−1.54692	1.77839	0.86984
North	Lisbon	0.028065	−3.96590	1.76839	2.24266
North	Alentejo	0.035413	−5.28201	1.77168	2.98136
North	**Algarve**	**0.049519**	**−8.62528**	**1.83095**	**4.71083**
Center	Lisbon	0.016079	−2.41897	1.76494	1.37057
Center	Alentejo	0.023427	−3.73509	1.76824	2.11233
Centro	**Algarve**	**0.037533**	**−7.07836**	**1.82762**	**3.87300**
Lisbon	Alentejo	0.007348	−1.31612	1.75818	0.74857
Lisbon	Algarve	0.021454	−4.65939	1.81789	2.56308
Alentejo	Algarve	0.014106	−3.34327	1.82109	1.83586

Allelic Diff: Difference of the allelic frequency in the two regions; Transf. Diff: Transformed difference of the allelic frequency, based on the angular transformation of each proportion, in the two regions; SE: standard error of the difference; q: Tukey statistics q.

tribution of the H63D mutation. Statistically significant differences were observed in the C282Y allelic frequencies between the global North (comprising the North and the Center regions) and the global South (comprising the Alentejo and the Algarve) (χ^2=3.841, $P<0.01$ Fisher's exact test).

Discussion

The present study demonstrates the existence of significant regional differences in the frequency of the C282Y mutation and a homogeneous frequency of the H63D mutation in Portugal. The results are in accordance with previously reported differences between biochemical parameters of the iron status in the normal population.[15] The highest allelic frequency of the C282Y mutation was observed in the North (0.058), approaching allelic frequencies reported in Northern European countries (reviewed in Lucotte,[20] Merryweather-Clarke,[21] Porto and De Sousa,[7] Fairbanks[22]), in contrast with the frequency in the Algarve (0.009), which is similar to that reported in Italy.[10,23] The allelic frequency of the H63D mutation observed in this study (0.15–0.20) is among the highest reported in Europe. Similar frequencies were seen in Spain (0.22)[11] with the highest frequency reported in Basques (0.30).[13]

The regional differences in the C282Y and the constancy of the H63D allelic frequencies raise two questions: (1) The putative age of the two *HFE* mutations; (2) The possible evolutionary significance of the differences observed in the C282Y allele frequency.

The findings are compatible with the notion of the recent origin of the C282Y mutation[24,25] and of an older origin for the H63D mutation as suggested by Risch[26].

The strategic localisation of Portugal between the Atlantic and the Mediterranean places the country at the convergence of many population settlements, coming from different parts of Europe and Africa.[27] These settlements, however, did not have the same influence in the whole country. A geographical and cultural boundary could be defined between the north and south, documented by archeological, ethnographical and linguistic records.[28] This is further supported by more recent studies of the European or African origin of Portuguese cattle breeds.[29]

Simon *et al*[30] postulated that the geographical distribution of hemochromatosis in the world was similar to the migration pattern of Celts, a postulate discussed more recently by Lucotte.[20] The occupation of the Celts in Portugal occurred in the 6th century BC and it is believed that the influence of the Celts was predominant in the North.[28] A strong founder effect could thus be an explanation for the marked differences observed. However, it goes against the putative age estimated to be on average 60 generations.[24,25] Considering that one generation corresponds to about 20 years, the C282Y mutation should have occurred 1200 years ago, which is more recent than the early Celtic occupation of Portugal. Another possible explanation for the distribution of the C282Y mutation could be the Nordic/Suevian occupation and settlement that occurred between the 5th–6th century only in the North of the country.[28] A possible later Viking origin has been discussed by others.[21,31]

The frequency of the H63D mutation did not vary between regions supporting an older origin of this mutation. Similarly to the present result, a reported study of 101 Portuguese unrelated males (53 from the north and 48 from the south) studied for 18 nuclear polymorphic markers, showed that 16 out of the 18 markers, had no differences in allelic frequencies between North and South suggesting a genetic homogeneity of the country.[32]

Thus, the high C282Y allelic frequency in the North supports the notion of acting selective forces.[25,33–35] The C282Y heterozygous subjects could be protected from iron deficiency when dietary iron intake is limited. In the case of the females, they could be protected from blood loss due to menstruation and multiple pregnancies. Interestingly, in the population from the North of Portugal a very high average number of pregnancies (>10 children) was reported in females older than 40 years.[36] In addition one could speculate that a mutation that favoured iron overload could be negatively selected in areas of high incidence of malaria such as European regions round the Mediterranean.[37] In Portugal, the highest mortality rate due to malaria was observed in the South.[38] Similar North/South discrepancies have been reported in France[39] and Italy.[23,40]

In conclusion, in addition to their interest for European population genetics, the present and similar results in other countries are a reminder of the need to take into account regional differences in the design of strategies for population screening of hereditary hemochromatosis in Europe and/or in countries with populations of European descent.

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