RESEARCH NOTE

Intragenic haplotype analysis of common HFE mutations in the Portuguese population

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Introduction

Most individuals with hereditary haemochromatosis are homozygous for the *HFE* gene mutation p.C282Y, and mutations p.H63D and p.S65C are associated with a mild iron overload phenotype. This work aims to investigate the *HFE* intragenic haplotype background associated with p.C282Y, p.H63D and p.S65C mutations in the Portuguese population by using seven intragenic single-nucleotide polymorphisms (SNPs). Ninety-eight subjects of Portuguese origin, previously diagnosed for the most common *HFE* gene mutations, were analysed and intragenic haplotypes were derived. Mutation p.C282Y (chr=56) was associated with one single haplotype GCTGTGC, and p.H63D (chr=81) was associated with two different haplotypes, GCCGTAC (66.7%) and CGCGTAC (33.3%). The p.S65C variant (chr=9) was found in the context of the single haplotype GCCGCAC. This study highlight, for the first time, the intragenic haplotypes associated with common HFE mutations in the Portuguese population.

Hereditary haemochromatosis (HH; OMIM# 235200), an autosomal recessive disorder caused by increased iron absorption, is one of the most common genetic diseases among subjects of European origin, affecting approximately 1 in 300 individuals of northern European descent (Adams 2000). The majority of hereditary haemochromatosis cases in Europe are due to mutations in the *HFE* gene (6p22.2), that encodes an HLA class-I-type protein important in iron regulation (Feder *et al.* 1996). Most haemochromatosis patients (80–90%) are homozygous for the *HFE* gene mutation c.845G>A (p.C282Y); two common missense mutations, c.187C>G (p.H63D) and c.193A>T (p.S65C), are associated with a mild iron overload phenotype (Feder *et al.* 1996; Sánchez *et al.* 1998; Merryweather-Clarke *et al.* 2000).

The allele distribution of p.C282Y in European countries correlates with haemochromatosis, and a decreasing gradient is observed from northern (highest frequencies between 6% and 14% observed in Ireland, United Kingdom, Scandinavia and France) to southern Europe, with the lowest allele frequencies in southeast Europe (<2%) (Murphy *et al.* 1998; Sánchez *et al.* 1998; Merryweather-Clarke *et al.* 2000; Lucotte and Dieterlen 2003). This p.C282Y European allele distribution, in addition to the observed similar pattern of associated haplotypes using HLA-class I alleles, external *HFE* microsatellites or intragenic SNPs, suggested for a single ancestral occurrence of the c.845G>A (p.C282Y) mutation <100 generations ago in the Celtic populations of mainland Europe, with a spread to west and north by population movements (Lucotte and Dieterlen 2003). Additionally, several reports have raised discussions regarding a p.C282Y Irish Celtic origin with a further spread by the Viking migrations (Olsson *et al.* 2010) or even a Viking origin (Merryweather-Clarke *et al.* 2000). Mutation c.187C>G (p.H63D) is worldwide distributed, showing 10–20% variable allele frequency in nearly all European populations, and also appearing in North Africa, the Middle East and Asia, supporting for an older origin of this mutation (Rochette *et al.* 1999; Ezzikouri *et al.* 2008; Reish *et al.* 2010; Dhillon *et al.* 2012; Karaca *et al.* 2013). The higher frequencies are in countries around the Mediterranean Sea (∼20%) (Sánchez *et al.* 1998; Merryweather-Clarke *et al.* 2000). For c.187G chromosomes, haplotype analysis revealed higher diversity consistent with conclusions that mutation p.H63D have originated earlier than p.C282Y or it is a recurrent mutation that occurred more than once on multiple ancestral haplotypes

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(Rochette *et al.* 1999; Yang *et al.* 2011). Variant c.193A>T (p.S65C) is present in ∼1.5% of the European population and was also observed in the context of different haplotypes (Mikhailova *et al.* 2010; Yang *et al.* 2011).

At least seven *HFE* intragenic SNPs have been analysed in different reports across different populations evidencing the evolutionary aspects for the three main risk alleles for iron overload p.C282Y, p.H63D and p.S65C (Rochette *et al.* 1999; Mikhailova *et al.* 2010; Yang *et al.* 2011; Dhillon *et al.* 2012). In the Portuguese population, the haemochromatosis *HFE* gene mutation p.C282Y was found at frequencies of 0.9–5.8%, evidencing regional differences in distribution across the mainland territory, in general agreement with the overall tendency for a north–south decreasing gradient (Cardoso *et al.* 2001). Mutation p.H63D was found at frequencies of 17–20%, with a homogeneous geographic distribution across the country (Cardoso *et al.* 2001). Although analysis of linkage disequilibrium (LD) between *HFE* mutations and *HLA* alleles or haplotypes was performed in the Portuguese population (Porto *et al.* 1998; Cardoso *et al.* 2002; Couto *et al.* 2003), p.C282Y, p.H63D and p.S65C associated haplotypes remain to be established using *HFE* intragenic SNPs. Thus, the aim of this study was to investigate the haplotype background of the three most common *HFE* mutations in Portuguese subjects using seven common intragenic polymorphisms.

Methods

Samples

A group of 98 subjects of Portuguese origin, previously diagnosed for the common *HFE* mutations at the Department of Haematology of the Centro Hospitalar e Universitário de Coimbra (CHUC) (Coimbra, Portugal), in the context of iron overload studies, were recruited: 11 homozygous for p.C282Y, 19 homozygous for p.H63D, 15 compound heterozygous for p.C282Y/p.H63D, 19 heterozygous for p.C282Y, 25 heterozygous for p.H63D, six heterozygous for p.S65C and three compound heterozygous for p.S65C/p.H63D.

Genotyping

Two SNPs in the 5′UTR *HFE* region, −1206C>G (rs1800 702) and −467G>C (rs2794720) relative to the ATG initiation codon, four SNPs located within intronic *HFE* regions near splice site boundaries, IVS2(+4)T>C (rs2071303), IVS4(+48)G>A (rs1800758), IVS4(−44)T>C (rs1800708) and IVS5(−47)G>A (rs1572982), and the polyA+5C>T (rs12346) SNP located at the 3′UTR *HFE* region, were analysed by PCR-RFLP. The *HFE* gene regions that include the studied SNPs were PCR-amplified using previously

Table 1. Primer sequences, restriction endonucleases and digested products for genotyping.

SNP ID	T_a (°C)	Primer sequence	PCR product (bp)	Restriction enzyme	Digestion product (bp)	Reference
rs1800702 −1206C>G	54	5′-GATCCTTTAACCGAGGAGAT-3′ 5′-CACTGGCCCACCTAAAT-3′	567	*Bbv*I	G: 511, 56 C: 567	Rochette *et al.* (1999)
rs2794720 −467G>C	60	5′-AAAGTTTGTGAAACACTTGTCAGA-3′ 5′-GCTTCGCAATGTTCTGATCT-3′	250	*Hpy*CH4IV	C: 201, 49 G: 250	Yang *et al.* (2011)
rs1799945 c.187C>G (p.H63D)	58	5′-GCTACGTGGATGACCAGCTGTACG-3′ 5′-CAGCTGTTTCCTTCAAGATGCAT-3′	267	*Mbo*I	C: 136, 99, 32 G: 136, 131	Mikhailova *et al.* (2010)
rs1800730 c.193A>T (p.S65C)				*Hin*fI	A: 151, 69, 41, 6 T: 151, 110, 6	
rs2071303 IVS2(+4)T>C				*Rsa*I	T: 246, 21 C: 170, 76, 21	
rs1800562 c.845G>A (p.C282Y)	60	5′-ACCAGGGCTGGATAACCTTGG-3′ 5′-GACTAGGGTGCCAGACGGTGA-3′	286	*Rsa*I	A: 232, 29, 25 G: 261, 25	Mikhailova *et al.* (2010)
rs1800758 IVS4(+48)G>A				*Mse*	G: 238, 48 A: 148, 90, 48	
rs1800708 IVS4(−44)T>C				*Hae*III	T: 213, 73 C: 142, 73, 71	
rs1572982 IVS5(−47)A>G	54	5′-TTTCCAGATGAGAGATAATGGTTCT-3′ 5′-TGGGGAAATCTTTTTGAGGA-3′*	360	*Nla*IV	G: 208, 102, 50 A: 208, 152	Mikhailova *et al.* (2010)
rs12346 PolyA+5C>T	54	5′-AAAGCTGTTATTTAATTAGCCAGTGA-3′* 5′-AAATGTCTCCAAAATGACAAAGC-3′*	187	*Rsa*I	T: 187 C: 100, 87	–

SNP ID, single-nucleotide polymorphism identification; T_a, annealing temperature; PCR product, polymerase chain reaction product. References (REF) are relative to primers; *Primers newly designed.

described primers (Rochette *et al.* 1999; Mikhailova *et al.* 2010; Yang *et al.* 2011, see table 1). SNPs were genotyped using BbvI for −1206C>G, HpyCH4IV for −467G>C, RsaI for IVS2(+4)T>C and polyA+5C>T, MseI for IVS4(+48) G>A, HaeIII for IVS4(−44)T>C and NlaIV for IVS5(−47) G>A (see table 1 for restriction-enzyme digested PCR products). To assess genotype reproducibility, 10% of random samples were selected and regenotyped by direct sequencing using the dideoxy chain termination reaction and the ABI Prism 3130 genetic analyzer (Applied Biosystems, Forster City, USA). Written informed consent was obtained from all the analysed subjects.

Statistical analysis

Haplotypes based on the seven intragenic *HFE* SNPs and mutations c.845G>A, c.187C>G and c.193A>T, were inferred using the ELB algorithm implemented in the software Arlequin, ver. 3.11 (http://cmpg.unibe.ch/software/arlequin3/) (Excoffier and Schneider 2005). Haplotype networks were constructed using the program NETWORK 4.6.1.2 (http://www.fluxus-technology.com/) using the median-joining algorithm and default parameters.

Results and discussion

The intragenic *HFE* haplotype analysis using the seven SNPs enables identification of 15 different haplotypes, four of them associated with the three common *HFE* mutations, c.845G>A, c.187C>G and c.193A>T (table 2). A network was constructed showing the relationships between 15 *HFE* haplotypes found in the Portuguese population, as also the haplotype backgrounds associated with each *HFE* mutation, c.845G>A, c.187C>G and c.193A>T (figure 1).

Considering the p.C282Y mutation, all the 56 c.845A carrier chromosomes were associated with one single haplotype GCTGTGC (H1 in table 2; figure 1). This haplotype is same as that found in a previous study by Yang *et al.* (2011) in Brittany (France) for five SNPs (underlined), CTGTG. Using the three most common studied SNPs, IVS2(+4)T>C, IVS4(−44)T>C and IVS5(−47)G>A, the observed TTG haplotype in the Portuguese population is the same that was previously found in the other populations of European origin (Beutler and West 1997; Mikhailova *et al.* 2010; Yang *et al.* 2011). This p.C282Y complete LD with a single intragenic haplotype argues with the common accepted notion that this mutation occurred once on a progenitor haplotype, and spread throughout all the European populations.

Regarding p.H63D mutation, the c.187G carrier chromosomes were associated with two different haplotypes GCCGTAC (66.7%) and CGCGTAC (33.3%) (H2 and H3 in table 2; figure 1) in accordance with previous results in the population of Brittany (France) (Yang *et al.* 2011) where the same two c.187G associated haplotypes CCGTA (79.8%) and GCGTA (20.2%) for five SNPs (underlined) were found. These two haplotypes diverge for the −1206C>G and −467G>C variations, found in complete LD in the sampled Portuguese population. The existence of two p.H63D associated haplotypes may suggest that the c.187C>G change occurred twice on different ancestral haplotypes (figure 1), as these two haplotypes, GCCGTAC and CGCGTAC, were also found in the normal chromosomes at frequencies of 4% and 2%, respectively (table 1). Alternatively, c.187C>G change may have occurred once in one ancestral haplotype,

Table 2. *HFE* haplotype analysis of a total of 98 subjects with *HFE* mutations including 11 homozygous and 19 heterozygous p.C282Y, 19 homozygous and 25 heterozygous p.H63D, 15 compound heterozygous p.C282Y/p.H63D, six heterozygous p.S65C and three compound heterozygous p.S65C/p.H63D.

ID	Haplotype	rs1800562 c.845G>A p.C282Y chr=56	rs1799945 c.187C>G p.H63D chr=81	rs1800730 c.193A>T p.S65C chr=9	Wild type chr=50
H1	GCTGTGC	56 (1.000)			8 (0.16)
H2	GCCGTAC		54 (0.667)		2 (0.04)
H3	CGCGTAC		27 (0.333)		1 (0.02)
H4	GCCGCAC			9 (1.000)	3 (0.06)
H5	CGTGTGT				13 (0.26)
H6	CGTATAC				8 (0.16)
H7	CGTGTAT				4 (0.08)
H8	GCTGTGT				3 (0.06)
H9	CGTGTAC				2 (0.04)
H10	GCTGTAC				1 (0.02)
H11	CGTATGC				1 (0.02)
H12	GCTATAC				1 (0.02)
H13	CGCGCGC				1 (0.02)
H14	CGTATAT				1 (0.02)
H15	CGTATGT				1 (0.02)

SNP sequence as follows: −1206C>G/−467G>C / IVS2(+4)T>C/IVS4(+48)G>A / IVS4(−44)T>C/ IVS5(−47)A>G / PolyA+5C>T.
ID, haplotype identification; chr, chromosome.

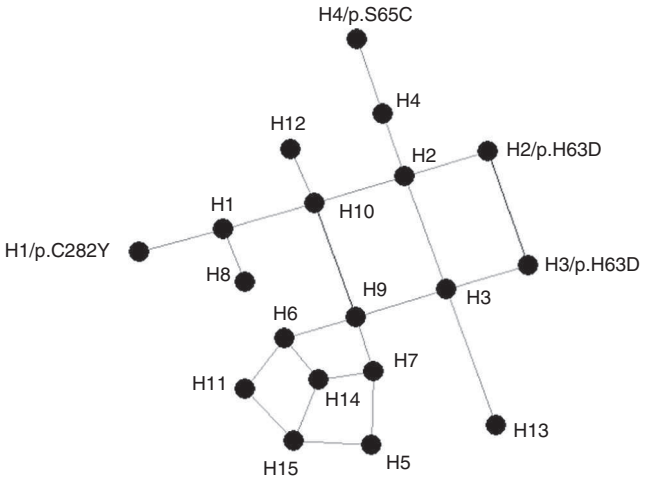

Figure 1. Median-joining network of the 15 *HFE* derived haplotypes found in the Portuguese population, shows also the haplotype backgrounds associated with each *HFE* mutation: c.845G>A (p.C282Y), haplotype H1 (GCTGTGC); c.187C>G (p.H63D), haplotypes H2 (GCCGTAC) and H3 (CGCGTAC); c.193A>T (p.S65C), haplotype H4 (GCCGCAC). Allelic sequences of haplotypes H1 to H15 are as shown in table 2.

probably the most common GCCGTAC, and the two different associated haplotypes could have arisen through a recombination event between loci −467 and IVS2(+4). For the three most common studied *HFE* SNPs, IVS2(+4)T>C, IVS4(−44)T>C and IVS5(−47)G>A, only the CTA haplotype was found associated with c.187G chromosomes in the Portuguese population, the same that was also reported by Yang *et al.* (2011) in Brittany, Mikhailova *et al.* (2010) in Russia and Beutler and West (1997) in American–European descent subjects. In the same way, a study in the Indian population for the p.H63D mutation (allele frequency ranging from 9.1% to 13.9%) revealed only the CTA European associated haplotype, which was interpreted as suggesting that p.H63D mutation has been introduced in India by population admixture with European chromosomes (Dhillon *et al.* 2012). On the other hand, in Sri Lanka, where the allele frequency is about 10%, c.187G mutation was found associated with three different haplotype backgrounds, TTG, TTA and CTG, suggesting that the c.187C>G change may have arisen more than once in Sri Lanka, independently from the mutation in Europe (Rochette *et al.* 1999).

Considering the p.S65C mutation, the nine c.193T chromosomes were found in the context of the single haplotype GCCGCAC (H4 in table 2; figure 1), different from the haplotype observed in Brittany CCGTA assuming five SNPs (underlined) (Yang *et al.* 2011). If only the three common loci IVS2(+4)T>C, IVS4(−44)T>C and IVS5(−47)G>A, were considered, the CCA haplotype found in Portuguese population has the same allele distribution to that found by Mikhailova *et al.* (2010) in Russia, but different from the CTA haplotype described by Yang *et al.* (2011) in Brittany.

In conclusion, this study highlight for the first time the intragenic haplotypes associated with common *HFE* mutations in the Portuguese population and the obtained results are compatible with those previously reported in other European populations. Mutation p.C282Y was found associated with one single haplotype, the same was previously observed in the European populations, which is compatible with the widely accepted notion of a Celtic or Nordic origin of the p.C282Y mutation. The p.H63D mutation was found associated with two different haplotypes suggesting the occurrence of this common *HFE* mutation on two different ancestral haplotypes or for recombination events occurring in the *HFE* gene region. Finally, the p.S65C mutation was found associated with one single haplotype compatible with the haplotype described in Russia, but different from that described in Brittany. This different genetic background for the c.193A>T (p.S65C) mutation could reflect specific demographic events in the region of Brittany (France) mainly inhabited by Celtic tribes during the prehistorical period and considered as one of the six Celtic nations.

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