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ORIGINAL ARTICLE

Non-classical hereditary hemochromatosis in Portugal: novel mutations identified in iron metabolism-related genes

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Abstract The most frequent genotype associated with Hereditary hemochromatosis is the homozygosity for C282Y, a common *HFE* mutation. However, other mutations in *HFE*, transferrin receptor 2 (*TFR2*), hemojuvelin (*HJV*) and hepcidin (*HAMP*) genes, have also been reported in association with this pathology. A mutational analysis of these genes was carried out in 215 Portuguese iron-overloaded individuals previously characterized as non-C282Y or non-H63D homozygous and non-compound heterozygous. The aim was to determine the influence of these genes in the development of iron overload phenotypes in our population. Regarding *HFE*, some known mutations were found, as S65C and E277K. In addition, three novel missense mutations (L46W, D129N and Y230F) and one nonsense mutation (Y138X) were identified. In *TFR2*, besides the I238M polymorphism and the rare IVS5 −9T→A mutation, a novel missense mutation was detected (F280L). Concerning *HAMP*, the deleterious mutation 5'UTR −25G→A was found once, associated with Juvenile Hemochromatosis. In *HJV*, the A310G polymorphism, the novel E275E silent alteration and the novel putative splicing mutation (IVS2 +395C→G) were identified. In conclusion, only a few number of mutations which can be linked to iron overload was found, revealing their modest contribution for the development of this phenotype in our population, and suggesting that their screening in routine diagnosis is not cost-effective.

Keywords Hereditary hemochromatosis · HFE · non-C282Y · Population screening

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Introduction

Hereditary hemochromatosis (HH) is an autosomal recessive disorder of adult-onset, common among Caucasians of Northern European ancestry, which leads to iron overload in several organs, if left untreated. Molecular studies have shown that HH is prevalently due to a founder missense mutation, C282Y, in the *HFE* gene on chromosome 6p21.3 [1]. Although C282Y in the homozygous condition remains the most frequent patients' genotype, other *HFE* mutations such as H63D and S65C have been described in compound heterozygosity with C282Y [1, 2]. In Southern Europe, a large percentage of individuals (35–45%) with HH phenotypes are neither C282Y homozygous nor C282Y/H63D compound heterozygous. Other very rare or private *HFE* variants have

been reported in affected individuals, contributing to the HH genetic heterogeneity [3–10]. Moreover, an association of HH with other genes, such as the transferrin receptor 2 (*TFR2*) [11–16], the hemojuvelin (*HJV*) [17–25] and the hepcidin (*HAMP*) [14, 26–31] genes, has also been documented. These latter two genes are mainly associated with the juvenile form of the disease (Juvenile Hemochromatosis, JH). The purpose of the current study was to search for uncommon *HFE*, *TFR2*, *HJV*, or *HAMP* mutations liable to explain the iron overload phenotype in Portuguese individuals previously characterized as non-C282Y or non-H63D homozygous and non-compound heterozygous. The result of this study will allow clarifying the importance of these genes in the routine HH genetic diagnosis in the Portuguese population.

Materials and methods

Selection of participants

The studied population consisted of 215 patients referred to the Human Genetics Centre of the National Institute of Health Dr. Ricardo Jorge in Lisbon, Portugal, for HH genetic testing. The criteria for patient's inclusion were to be adult, not related and presenting serum ferritin level >400 ng/ml or transferrin saturation >50% (unless currently treated by phlebotomy). All these patients were previously diagnosed as non-C282Y or non-H63D homozygous and non-compound heterozygous.

Fifty control samples from unrelated healthy individuals were also studied. Investigations were undertaken with the written informed consent of all participants.

Molecular studies

Polymerase chain reaction (PCR) amplification of *HFE* exons 2, 3 and 4, and of *TFR2* exons 2, 4, 5 and 6 (including intron/exon boundaries) was performed for each patient's DNA, as described by Le Gac et al. 2001 [7] and Roetto et al. 2001 [12]. Screening for mutations in PCR-amplified DNA was performed by using both single-strand conformation polymorphism (SSCP) and denaturing high performance liquid chromatography (dHPLC). Purified PCR products presenting altered profiles were directly sequenced using the ABI Prism BigDye Terminator v1.1 Cycle Sequencing Kit in the ABI Prism 3130xl Genetic Analyzer (Applied Biosystems). Codifying regions of *HJV* and *HAMP* genes were screened by dHPLC as described or were directly sequenced [18, 19, 27, 29].

In silico studies

Potential splice signals of mutated DNA sequences were analyzed using the SpliceView software (http://l25.itba.mi.cnr.it/~webgene/wwwspliceview.html) and multiple sequence alignment of HFE protein from mouse, rat, human and dog was accomplished by using the ClustalW *v1.82* software (http://www.ebi.ac.uk/clustalw).

Results and discussion

In this study, the mutational screening of *HFE* and *TFR2* was performed by using two different methodologies, SSCP and dHPLC. The analysis of *HFE* exon 2 revealed five abnormal profiles. The respective sequencing allowed the identification of the known S65C in compound heterozygosity with H63D in two individuals, in compound heterozygosity with C282Y in other individual, and in another one in the heterozygous condition. The other abnormal profile was due to a T→G transversion in heterozygosity at codon 46 (Fig. 1a). Consequently, leucine is replaced by tryptophan originating a novel mutation, L46W. It was found in a 45-year-old male presenting a transferrin saturation=68% and ferritin=444 ng/ml.

Although three abnormal dHPLC profiles of HFE exon 3 were observed, only two of them were detected by SSCP. One was due to a G→A transition at codon 129 (Fig. 1b), resulting in an aspartic acid to asparagine alteration and, consequently to the novel D129N mutation. It was detected in a 35-year-old man in compound heterozygosity with H63D. The patient presented transferrin saturation=79.6% and ferritin=135 ng/ml.

Both L46W and D129N patients' families were not available to be studied, so it was not possible to observe a familiar segregation associated to the iron overload phenotype. The L46W mutation is localized near the flexible HFE α1 domain loop Q40–S45, which interacts with helix 1 of the transferrin receptor 1 (TfR1) helical domain [32, 33]. On the other hand, the D129N mutation modifies a residue located in HFE α2 domain which interacts with helix 2 of TfR1. Therefore, these two novel mutations could affect HFE affinity for TfR1 and consequently might interfere with its proper functioning. Moreover, it was observed that both L46 and D129 are highly conserved residues (Fig. 2a and b), so they should be important for protein function. Splicing analysis suggested that both mutations do not affect splicing.

As L46W was found only in heterozygosity, it is unlikely that this mutation, by itself, justifies the patient iron overload. In addition, this individual has alcoholic habits that probably also contribute to his iron burden. Concerning the D129N/H63D patient, attending to the novel mutation putative functional effects and to the absence of known external contributor factors, it might be a pathologic genotype.

Sequencing of the other two *HFE* exon 3 abnormal profiles revealed two novel alterations at tyrosine 138: C→T and C→G, giving rise to Y138Y (only detected by dHPLC) and Y138X, respectively. The former alteration that does not change the amino acid, was detected in heterozygosity in a

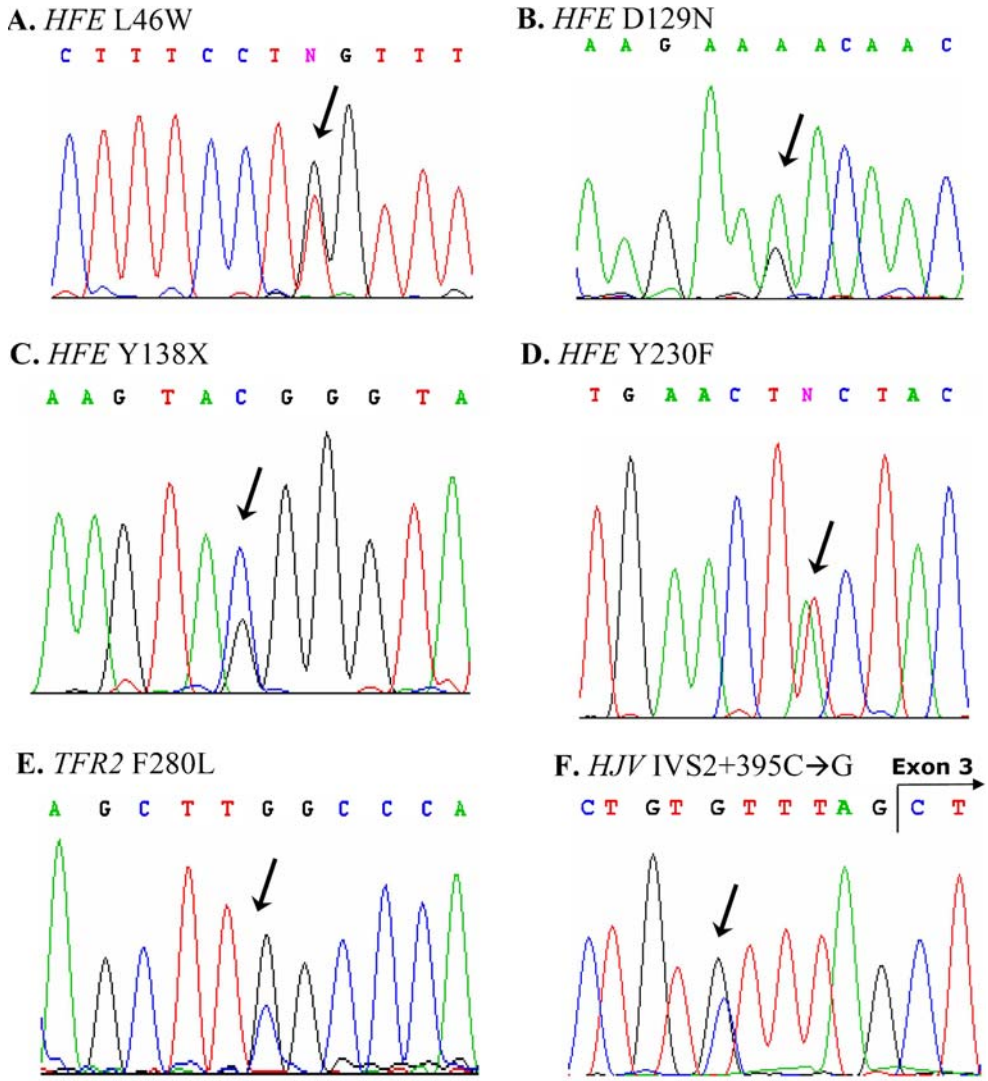

Fig. 1 Sequencing results showing the mutations found in the *HFE*, *TFR2* and *HJV* genes

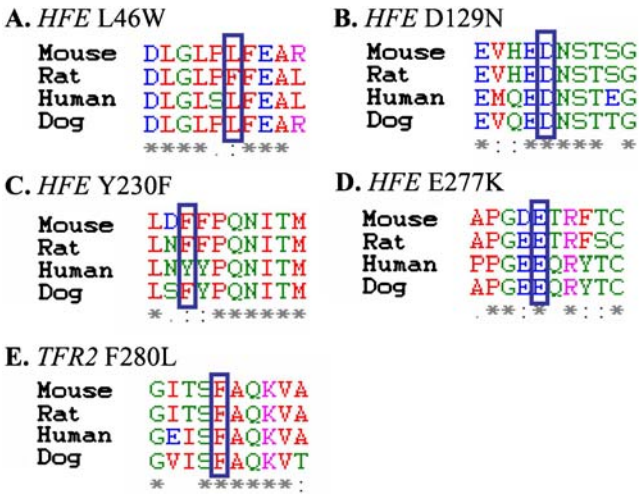

Fig. 2 Multiple sequence alignment of HFE and TFR2 protein from mouse, rat, human and dog using the *ClustalW v1.82* software. Considered residues are indicated by a rectangle; identical residues by an asterisk (*); conserved substitutions by a colon (:) and semi-conserved substitutions by a dot (.)

69-year-old man who presents transferrin saturation=45% and ferritin=1466 ng/ml. This mutation causes a minor alteration of the consensus value of a potential donor site (from 82 to 79). However, because this site is not commonly used, it is unlikely that this mutation affects splicing. So it may be an alteration without any pathologic significance and does not justify the patient's iron overload.

On the opposite, the nonsense mutation (Y138X) (Fig. 1c) was detected in compound heterozygosity with C282Y in a 63-year-old man presenting transferrin saturation = 90% and ferritin=1,000 ng/ml. This novel mutation originates a premature stop codon and consequently the correspondent mRNA will be committed to the nonsense-mediated mRNA decay mechanism. Therefore, this patient genotype (Y138X/C282Y) entirely justifies his HH phenotype.

Two altered *HFE* exon 4 profiles were found. Sequencing revealed in one case a novel heterozygous A→T transversion at codon 230 (Fig. 1d), substituting a tyrosine for a phenylalanine (Y230F). The patient's family study revealed that

although his father is a compound heterozygous for Y230F and H63D, he presents normal iron parameters (Table 1). Y230F alters a residue positioned on a β-strand of the α3 domain which binds to β2-microglobulin [32, 33]. However, the Y230 position is originally occupied by a phenylalanine in some species (Fig. 2c), suggesting that tyrosine at position 230 may not be essential. Also, this mutation induces a small alteration of a potential acceptor site consensus value (from 81 to 85) but probably without consequences. Taking into consideration all these facts, it is unlikely that this mutation is contributing for the iron overload in this patient.

The other abnormal profile found in *HFE* exon 4 was due to the known E277K [34, 35] mutation, found in compound heterozygosity with H63D in a patient. His family study showed that his brother presents the same genotype and also a high serum ferritin level (Table 2). The mutation does not alter splicing signals and E277 is highly conserved between species (Fig. 2d). Although the E277K has been previously described as a neutral polymorphism [34], in this study, it was detected in compound heterozygosity with H63D in two brothers both presenting altered iron parameters. Due to its HFE α3 domain localization, this amino acid alteration might perturb the binding to β2-microglobulin, affecting HFE correct processing. Altogether these facts suggest some deleterious effect of this mutation, but only functional studies could determine its real significance.

The screening of *TFR2* revealed that three out of the 215 patients had altered dHPLC profiles. The direct sequencing identified one heterozygous transversion C→G at codon 238, which causes an amino acid substitution from isoleucine to methionine, I238M. This alteration was already reported as a polymorphism by Lee et al, 2001 [13].

Another *TFR2* variant corresponds to a novel C→G mutation at codon 280 (Fig. 1e) which originates a phenylalanine to leucine substitution (F280L). It was found in double heterozygosity with H63D in a 29-year-old man presenting a transferrin saturation=80% and ferritin=111 ng/ml. This mutation does not alter splicing signals and F280 is a highly conserved residue (Fig. 2e). It is located in the apical region of TFR2, so the amino acid change might perturb protein function and, consequently, it's binding to transferrin. Considering the patient's alcoholic habits, the cause of his iron overload is probably the combined action of genetic and environmental factors. The F280L mutation requires functional studies to complete its characterization.

Table 1 Clinical data of the *HFE* Y230F family

	Propositus	Father	Mother	Brother
Age (years)	30	55	54	23
Sex	M	M	F	M
Cardiomyopathy	–	+	–	–
Diabetes mellitus	–	–	+	–
Iron (μg/dl)	162	76	69	63
Transferrin saturation (%)	75.8	35.8	26.3	32.1
Ferritin (ng/ml)	531	165	74	258
HFE genotype	Y230F/wt	Y230F/H63D	wt/wt	H63D/wt

wt Wild type, + presence, − absence

Table 2 Clinical data of the *HFE* E277K family

	Propositus	Brother	Sister I	Sister II
Age (years)	63	67	59	46
Sex	M	M	F	F
Cardiomyopathy	–	+	–	–
Iron (μg/dl)	185	115	77	30
Transferrin saturation (%)	60.7	39.5	25	9
Ferritin (ng/ml)	620	812	72	20.8
HFE genotype	E277K/H63D	E277K/H63D	H63D/wt	wt/wt

wt Wild type, + presence, − absence

The other *TFR2* abnormal profile found corresponded to the rare IVS5 −9T→A alteration [15]. It was detected in double heterozygosity with H63D in a 38-year-old man presenting transferrin saturation=51% and ferritin=186 ng/ml. This mutation produces a small reduction of the native acceptor splicing site consensus value (88 to 84) and its effect on splicing can be hypothesized.

The 5′UTR −25G→A (24) mutation was found in homozygosity in the *HAMP* gene in a 50-year-old man who presented, at the age of 28, a severe juvenile hemochromatosis phenotype (transferrin saturation=70%; cardiomyopathy, hepatomegaly, intense hepatic hemosiderosis) [36]. This mutation creates a new initiation codon at position +14 related to the cap site, which induces a shift of the open reading frame. Homozygosity for this mutation was previously reported as the cause of JH in two other individuals of Portuguese origin [31, 37]. As this third affected individual had a birth place located near the first one described (Matthes T, personal communication), probably they are related to the same *HAMP* mutational event.

Two of the *HJV* alterations found are apparently asymptomatic. The A310G is described as a polymorphism in ensEMBL (www.ensembl.org) and GeneCards® (www.genecards.org) databases. The other one, a G→A transition at codon 275, does not alter the amino acid residue (E275E) and does not seem to affect splicing. On the other hand, the third alteration observed, the IVS2 +395 C→G (Fig. 1f), was detected in heterozygosity in a 29-year-old man presenting transferrin saturation=59% and ferritin=661 ng/ml. It is located at six deoxyribonucleotides from the beginning of exon 3 and induces an alteration of the consensus value of the native acceptor site (from 89 to 85), which becomes lower than another potential splice site (86). Consequently, some competition between these two splice sites might

occur, altering the splicing variants produced. Even if the mutation deleterious effect is confirmed in future functional studies, it is improbable that its heterozygosity is the only cause of the individual iron overload.

All novel mutations found were searched for and not found in 50 healthy individuals, so they cannot be considered polymorphisms.

In conclusion, in this study, several alterations were found in *HFE*, *TFR2*, *HJV*, and *HAMP* genes, screened in 215 Portuguese individuals presenting iron overload, previously characterized as non-C282Y or non-H63D homozygous or compound heterozygous. However, only a few number of these mutations can be directly associated with HH or JH, revealing a modest contribution of the novel variants in this phenotype development, and suggesting that their screening in routine diagnosis is not cost-effective. Nevertheless, the identification and study of these novel mutations is important to enlarge the knowledge of the HH genetic heterogeneity. Additionally, their functional study can be significant for a better understanding of the correspondent proteins role in the iron metabolism and in HH development.

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