Next-generation sequencing of iron metabolism-related genes in Portuguese patients with iron overload: novel pathogenic genetic variants

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INTRODUCTION

Hereditary Hemochromatosis (**HH**) is an autosomal recessive disorder characterized by excessive intestinal iron absorption resulting in pathologically increased body iron stores. It is typically associated with a common *HFE* gene mutation (p.Cys282Tyr)(1). However, particularly in Southern European populations, around one third of the patients with primary iron overload do not present that genotype or the compound heterozygosity for the p.Cys282Tyr and the p.His63Asp mutations (2). In fact, in addition to *HFE*, other iron metabolism-related genes may be involved in HH development (3).

This study aimed to explore the use of the **Next-Generation Sequencing** (NGS) technology for rapid and simultaneous analysis of a panel of six iron metabolism-related genes (*HFE*, *TFR2*, *HJV*, *HAMP*, *SLC40A1,* and *FTL*) in 88 Portuguese patients with persistent iron overload, not exposed to environmental factors known to promote this phenotype, and a negative *HFE*-HH-first level genetic test.

METHODS

Eighty-eight iron overload Portuguese patients, negative for the common *HFE* risk genotypes were analysed by **NGS** for six iron metabolism-related genes (***HFE, TFR2, HAMP, HJV, SLC40A1 and FTL***).

A TruSeq Custom Amplicon kit (TSCA, by Illumina) was designed in order to generate 97 amplicons covering exons, intron/exon junctions and UTRs of the mentioned genes with a cumulative target sequence of 12115bp. Amplicons were sequenced in the MiSeq instrument (Illumina) using 250 bp paired-end reads. Sequences were aligned against human genome reference hg19 using alignment and variant caller algorithms in the MiSeq reporter software (Illumina).

Novel and very rare variants were validated by Sanger sequencing (Applied Biosystems) and their pathogenic significance were assessed by *in silico* studies (Polyphen-2, Human Splicing Finder).

RESULTS

A total of 1242 genetic alterations (corresponding to 55 different variants) were found by NGS in the 88 Portuguese non-classical HH patients. Fourteen of them are presented in **Table I**. These include three novel mutations in *TFR2* [two missense (p.Leu750Pro and p.Ala777Val) and one intronic splicing mutation (c.967-1 G>C)] (**Fig.1**), one novel missense mutation in *HFE* (p.Tyr230Cys), one private mutation at the 5'-UTR of *HAMP* (c.-25 G>A) which gives rise to a premature translation initiation codon, and two mutations in the Iron-Responsive Element (IRE) of *FTL* gene, c.-173C>G and c.-167C>A (**Fig.2**).

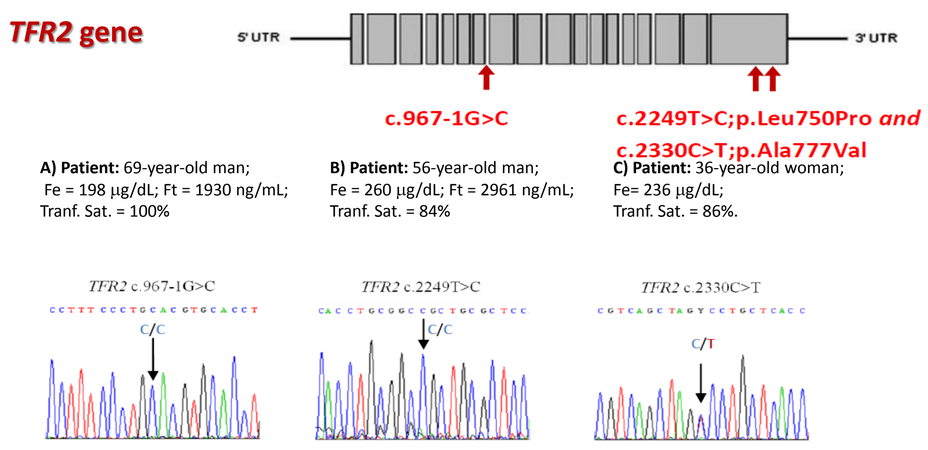

Fig. 1 - Sanger sequencing electropherograms with validation of the novel pathogenic variants detected by NGS in *TFR2* gene:
A) homozygosity for the ***TFR2:*c.967-1G>C**; **B)** homozygosity for the ***TFR2*:c.2249T>C, p.(Leu750Pro)**; **C)** heterozygosity for the ***TFR2:*c.2330C>T, p.(Ala777Val)** - this patient is a double heterozygous for the mentioned variant and the ***HFE*:c.187C>G,p.(His63Asp)**.

Iron-Responsive Element of FTL gene

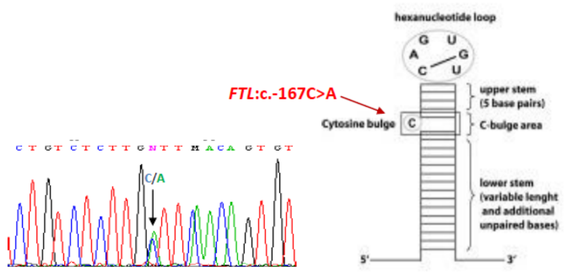

Fig. 2 - Sanger sequencing electropherogram showing the c.-167C>A variant in heterozygosity. It is located in the **IRE of *FTL* gene** and was found in segregation in a family in association with **hereditary hyperferritinemia with congenital cataracts.**

Table I – Novel or private genetic variants found by NGS in Portuguese patients with non-classical HH

Genomic location (hg19)	Gene	NGS allelic depth	Zygosity	NGS Qual	Genomic context	Nucleotide change	Amino acid change	Variant classification by *in silico* studies
Chr6:26092985A>G	*HFE*	430:455	het	9069.08	Exon 4	c.689A>G	p.(Tyr230Cys)	Missense; Possibly Damaging
Chr6:26092801T>C	*HFE*	57:66	het	1325.07	Intron 3	c.617-112T>C	-	Likely benign
Chr7:100229569C>G	*TFR2*	0:453	hom	10655.17	Intron 7	c.967-1G>C	-	Affects splicing; Possibly Damaging
Chr7:100218637A>G	*TFR2*	4:215	hom	4960.62	Exon 18	c.2249T>C	p.(Leu750Pro)	Missense; Probably damaging
Chr7:100218556G>A	*TFR2*	Case 1: 141:116 Case 2: 141:146	het het	2236.37 4408.53	Exon 18	c.2330C>T	p.(Ala777Val)	Missense; Probably damaging
Chr19:35773456G>A	*HAMP*	Case 1: 0:394 Case 2: 2:476	hom hom	15985.68 19146.68	5'UTR	c.-25G>A	-	Alters ORF; Pathogenic
Chr19:35773318G>A	*HAMP*	62:49	het	874.57	5'UTR	c.-92G>A	-	Likely benign
Chr1:145414708G>A	*HJV*	240:218	het	4285.30	5'UTR	c.-74C>T	-	Likely benign
Chr1:145415253G>A	*HJV*	47:44	het	814.49	Intron 3	c.1002-26C>T	-	Likely benign
Chr1:145415769A>G	*HJV*	59:62	het	1198.75	Exon 4	c.932T>C	p.(Asn129=)	Synonymous/ Likely benign
Chr2:190436342A>G	*SLC40A1*	242:180	het	3470.70	Intron 5	c.514+99T>C	-	Likely benign
Chr19:49468592C>G	*FTL*	39:36	het	653.68	IRE_ 5'UTR	c.-173C>G	-	Possibly Pathogenic
Chr19:49468598C>A	*FTL*	24:25	het	472.00	IRE_ 5'UTR	c.-167C>A	-	Pathogenic
Chr19:49469233T>G	*FTL*	138:164	het	3347.00	Intron 2	c.249+60T>G	-	Likely benign

DISCUSSION

The recent development of NGS allows practical, manageable, and cost-effective analysis of the non-classic HH cases, proving to have significant utility when conventional testing has failed to identify the underlying molecular basis of the disease. However, establishing the clinical relevance of NGS-detected variants for HH development remains a hard-working task, requiring further functional studies.

The identification and study of novel iron metabolism-related mutations are important steps forward to improve the knowledge of the HH genetic basis heterogeneity and of the pathophysiology of the different types of HH. Clinically, it is also important because whilst the treatment of the more common forms of iron overload is similar, differential diagnosis remains important in atypical cases in which specific treatment and/or monitoring options are recommended.

REFERENCES

(1) J. N. Feder, *et al*. A novel MHC class-I gene is mutated in patients with hereditary haemochromatosis. Nat. Genetics 13 (1996) 399-408.

(2) A. T. Merryweather-Clarke, *et al*. Geography of HFE C282Y and H63D. Genet. Test. 4 (2000) 183-198.

(3) B. Silva, P. Faustino. An overview of molecular basis of iron metabolism regulation and the associated pathologies. BBA 1852 (2015) 1347-1359.

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