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A novel homozygous stop-codon mutation in human *HFE* responsible for nonsense-mediated mRNA decay

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ABSTRACT

HFE-hemochromatosis (HH) is an autosomal disease characterized by excessive iron absorption. Homozygotes for H63D variant, and still less H63D heterozygotes, generally do not express HH phenotype. The data collected in our previous study in the province of Matera (Basilicata, Italy) underlined that some H63D carriers showed altered iron metabolism, without additional factors. In this study, we selected a cohort of 10/22 H63D carriers with severe biochemical iron overload (BIO). Additional analysis was performed for studying *HFE* exons, exon–intron boundaries, and untranslated regions (UTRs) by performing DNA extraction, PCR amplification and sequencing. The results showed a novel substitution (NM_000410.3:c.847C>T) in a patient exon 4 (GenBankJQ478433); it introduces a premature stop-codon (PTC). RNA extraction and reverse-transcription were also performed. Quantitative real-time PCR was carried out for verifying if our aberrant mRNA is targeted for nonsense-mediated mRNA decay (NMD); we observed that patient *HFE* mRNA was expressed much less than calibrator, suggesting that the mutated HFE protein cannot play its role in iron metabolism regulation, resulting in proband BIO. Our finding is the first evidence of a variation responsible for a PTC in iron cycle genes. The genotype–phenotype correlation observed in our cases could be related to the additional mutation.

Introduction

HFE-hemochromatosis (HH; OMIM#235200) is a genetic disorder characterized by excess of iron absorption, which progressively leads to multi-organ failure. The main clinical consequences, related to the ferrotoxicity, are hepatic cirrhosis, hepatocellular carcinoma, cardiomyopathy, diabetes, arthritis and hypogonadism [1,2]. The early biochemical expression of HH is characterized by iron increase (particularly serum ferritin) and the diagnosis relies on the liver biopsy associated with evidence of increased iron content [1–3]. After the identification of the *HFE* gene (OMIM*613609) on short arm of chromosome 6 at location 6p22.2 [4], the genetic test has become the gold standard for diagnosis and it is now widely available.

HFE coding region is formed by 1047 nucleotides that encode a 348 amino acid glycoprotein known as HFE. This transmembrane protein presents a structure very similar to the major histocompatibility complex (MHC) class I molecules. It competes with transferrin for binding to the transferrin receptor (TFR; OMIM*90010) [5,6]. Two extracellular domains, α1 and α2, sit on the top of the α3 domain, which spans the cellular membrane and binds to the beta-2-microglobulin (β2M) protein (OMIM*109700). HFE protein is formed by a signal peptide, a topical extracellular domain, a transmembrane helical region and a cytoplasmic domain. It plays a role in the iron regulation by forming complexes with (a) TFR1 (OMIM*190010), in case of iron deficiency or (b) TFR2 (OMIM*604720), the TFR1 liver homologue, in case of iron overload. HFE/TfR2 complex activates a signaling cascade resulting in the upregulation of hepcidin (OMIM*606464), ferroportin degradation (OMIM*604653) and, consequently, a decreased dietary iron uptake [7–9].

HH clinical expression occurs earlier in male (around the age of 40) and later in females, because of the protective effects of menstrual blood loss and pregnancies [10].

HFE protein mutation leads to iron and organ damage in the liver, heart and pancreas [2]. Worldwide about 20 different mutations have been identified in *HFE* gene [11,12], but the main mutations are known as C282Y (rs1800562; exon 4, NM_000410.3:c.845G>A; NP_000401.1:p.Cys282Tyr) and H63D (rs1799945; exon 2, NM_000410.3:c.187C>G; NP_000401.1:p.His63Asp) [12,13]. C282Y mutation is responsible for the lack of HFE-β2M association [14]; H63D variant leads to the lack of HFE–TFR complex [6–15].

Abbreviations: β2M, beta-2-microglobulin; HH, HFE-hemochromatosis; MHC, major histocompatibility complex; NMD, nonsense-mediated mRNA decay; PTC, premature translation-termination codon; qPCR, quantitative real-time PCR; TFR, transferrin receptor; UTRs, untranslated regions.

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The distribution of genotypes among iron loaded patients shows a prevalence of C282Y/C282Y subjects [16–19]. They are more than 90% in the UK, and more than 80% in Northern European countries; the percentage ranges from 60 to 83% in the USA [20] and it is equal to 64% in Italy [21]. A mild-moderate phenotype is associated to the compound heterozygosity condition (C28Y/H63D) [22]. H63D role in iron overload is controversial, but this condition less commonly develops iron overload [10,12,20] and is associated with extremely variable phenotypes [23]. In our previous study we firstly investigated the distribution of HH genotypes among iron overloaded patients belonging to the province of Matera (Basilicata, South Italy) based on the genetic testing describing a biochemical evidence of iron overload (serum ferritin value higher than 750 ng/ml) in H63D/wt individuals, in the absence of other factors related to iron overload [24]. The genetic test (Haemochromatosis Strip Assay StripAssayA by ViennaLab Diagnostic GmbH) covers 11 HFE gene mutations (V53M, V59M, H63D, H63H, S65C, Q127H, E168X, E168Q, W169X, C282Y, and Q283P), 4 transferrin receptor mutations (Y250X, E60X, M172K, and the AVA Q 594–597) and 2 ferroportin mutations (N144H and V162del). Routine clinical practice is affected by considering HH allelic heterogeneity and the existence of rare/private mutations, occasionally found in a small number of subjects [25]. Considering the spectrum of possible genetic variations and the need of early diagnosis, in order to optimize the treatment of some patients at risk of delayed appropriate treatment, we decided to evaluate the potential utility of additional genetic investigations. Therefore we retrospectively selected a cohort of patients with H63D heterozygosity and biochemical iron overload for additional studies. In this context we assessed the *HFE* mutational state in order to clarify the relationship between genotype and phenotype in a patient belonging to our cohort.

Patient and methods

We retrospectively identified a cohort of 22 patients with H63D heterozygosity and biochemical iron overload for additional studies. The patients were divided into two groups: the first one included 12 subjects with ferritin value lower than 750 ng/ml; within the second cluster resided 10 individuals whose ferritin level exceed 750 ng/ml (severe hyperferritinemia). We assessed the *HFE* mutational state in order to clarify the relationship between genotype and phenotype in this last cluster. The most significant data of the subjects belonging to the second group are reported in Table 1.

About the methods, the first step of molecular biology consisted in (a) DNA isolation, (b) primer design, (c) PCR (polymerase chain reaction) amplification, (d) *HFE* fragment sequencing and (e) bioinformatics analysis. DNA extraction was carried out by using a commercial kit (Nuclear Laser Medicine S.r.l.) according to the manufacturer's instructions. DNA was quantified by means of NanoDrop 1000 spectrophotometer (NanoDrop Technologies, Inc). On the basis of *HFE* RefSeq in NCBI database, PCR primers were designed by means of NCBI Primer-Blast in order to detect *HFE* exons, exon–intron boundaries, and 5′ and 3′ untranslated regions (UTRs). A phase of the optimization of PCR components and conditions was carried out. For the amplification, 25 μl of PCR reaction was used: 1.5 μl $MgCl_2$, each of dNTP 2 mM, 1 μl of specific primers, 0.4 U/μl of AmpliTaq Gold DNA polymerase in 10× PCR buffer (100 mM tris–HCl, pH 8.3, 500 mM KCl) (Roche Molecular Systems, Inc). The conditions of reaction were the following: (1) initial denaturation: 95 °C/7 min; (2) thermocycling: 94 °C/1 min; 58 °C/1 min; 72 °C/2 min (35 cycles); (3) final extension: 72 °C/10 min. Amplification products were analyzed by gel electrophoresis (1.5% agarose gel).

The next step consisted in *HFE* fragments sequencing. The bioinformatics analysis allowed to confirm the similarity between our sequences and *HFE* RefSeq, by means of BlastN (NCBI database) [26]; a multiple alignment was performed between patient sequences and control sequences (three healthy controls were included in the study) to identify conserved/nonconserved nucleotides, by means of ClustalW2 (EMBL-EBI database) [27]. In addition the *Mutation Surveyor* software was employed for DNA variant analysis [28,29]. If a novel mutation was identified, it was confirmed by a second step of amplification.

In addition, for assessing the *HFE* mRNA level, quantitative real-time PCR (qPCR) assay was carried out. First, the whole blood was collected by means of Tempus RNA tubes (Applied Biosystems). RNA isolation was achieved using Tempus Spin RNA Isolation Reagent Kit (Applied Biosystems), optimized in our laboratory. RNA was quantified using the NanoDrop Spectrophotometer 1000 (NanoDrop Technologies, Inc.) again. The reverse transcription of RNA into double-stranded cDNA was obtained by using the RETROscript Kit (Ambion), following the company instructions.

The qPCR was carried out by means of the thermal cycler Chromo4 (Continuous Fluorescence Detector) and the Optical Monitor 2.03.5 software (MJ Research, Inc.). We employed the Power SYBR Green PCR Master Mix (Applied Biosystems) in the following volumes: 5 μl of Power SYBR Green PCR Master Mix (2×), 1.5 μl of forward primer 10 mM, 1.5 μl of reverse primer 10 mM, 1 μl of template. The protocol was (1) 95 °C/10 min, (2) 38 cycles of 95 °C/30 s and 64 °C/1 min, and (3) 72 °C/1 min. Melting curves were generated after 38 cycles by heating the sample up to 95 °C for 15 s followed by cooling down to 60 °C for 15 s and heating the samples to 95 °C for 15 s. We applied SYBR Green as reporter molecule, β-actin as housekeeping gene, three wild-type subjects as calibrators, two gene replicates and $2^{-\Delta\Delta Ct}$ method [30] for data analysis, according to the Minimum Information for Publication of Quantitative Real-Time PCR Experiments (MIQE) [31]. After verifying that all gene amplification efficiency was comparable, we calculated the fold change ($2^{-\Delta\Delta Ct}$). In detail, firstly we determined average Ct values for *HFE* replicates and for controls (calibrators) and, after normalization with housekeeping, we calculated ΔΔCt as ΔCt of target gene − ΔCt of calibrator gene and we used the data for deriving the fold change value. Results were expressed as mean ± standard deviation. Student's *t* test was employed in order to calculate the statistical significance ($p < 0.05$). PCR specificity was assessed by melting curve analysis of amplification products for confirming the occurrence of specific amplification peaks and the absence of primer dimer formation. Gel electrophoresis (2% agarose) was also performed in order to verify the presence of single gel bands of predicted size.

Table 1
The most significant data of the patients belonging to the "severe hyperferritinemia" group.
Age, sex and serum ferritin value of the 10 subjects whose serum ferritin (SF) value is higher than 750 ng/ml.

Patients	Age (years)	Sex	SF average value (ng/ml)
1	35	Male	1035
2	41	Male	948
3	48	Male	989
4	26	Male	792
5	69	Male	1230
6	54	Male	957
7	33	Female	922
8	45	Male	875
9	51	Male	1154
10	55	Male	860

Results

HFE sequencing results underlined the presence of a variant not previously described in literature (GenBank Number: JQ478433) in 9/10 patients in the group of severe hyperferritinemia. We first identified this variation in the patient 1 of the cluster and we consequently extended the variant research to the whole group. For this reason the proband 1 was considered the case-model and we report about the subject, a 35-year-old Caucasian male coming from a small village of Basilicata, whose father (76 years old) showed severe