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H63D mutation in the HFE gene increases iron overload in β-thalassemia carriers

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Background and Objectives. Hereditary hemochromatosis (HH) is an autosomal recessive disorder of iron metabolism. The HFE gene implicated in this disorder has been identified on chromosome 6 (6p21.3). The most prevalent mutation in HH patients changes the 282 cysteine residue to tyrosine (C282Y). The role of a second mutation which changes the 63 histidine to aspartic acid (H63D) in iron overload has been controversial. The aim of this study was to evaluate the effect of the H63D mutation on the ferritin levels of β-thalassemia carriers.

Design and Methods. β-thalassemia carriers have a tendency to increase iron absorption because of mild anemia and slightly increased erythropoiesis. Differences in ferritin levels between homozygotes for H63D and wild type may indicate a modulator effect of the HFE mutation on iron absorption. We studied 152 healthy males, heterozygous for β-thalassemia. Serum ferritin was measured by chemiluminescence. H63D genotypes were determined by digestion of polymerase chain reaction (PCR) products with MboI restriction enzyme.

Results. Forty-five subjects were H63D heterozygotes and four subjects were H63D homozygotes. Ferritin levels were (mean±SD): 250±138 μg/L in homozygotes for the wild type H/H; 295±186 μg/L in H/D heterozygotes; and 389±75 μg/L in homozygotes for the mutation D/D. The difference in ferritin values between H/H and D/D is statistically significant (p=0.022).

Interpretation and Conclusions. β-thalassemia carriers who are homozygotes for the H63D mutation have higher ferritin levels than β-thalassemia carriers with the H/H genotype, suggesting that the H63D mutation may have a modulating effect on iron absorption.

Key words: hemochromatosis, HFE gene, H63D mutation, β-thalassemia.

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Mutations of the HFE gene have recently been reported to be responsible for hereditary hemochromatosis which is a common autosomal recessive disorder of iron metabolism that, without treatment, results in iron overload.[1] The majority of patients with hereditary hemochromatosis, more than 80% in North Europe, carry a unique missense mutation: a G to A transition at nucleotide 845, which changes amino acid 282 from cysteine to tyrosine (C282Y). The C282Y mutation, preventing the formation of a disulfide bond in the α3 domain and abrogating β2-microglobulin association as well as cell surface expression of the protein, may be sufficient to cause iron storage overload.[2] A second mutation, a C to G transversion at nucleotide 187, which changes amino acid 63 from histidine to aspartic acid (H63D), does not prevent β2-microglobulin association or cell surface expression[2] but, *in vitro* alters HFE's ability to reduce the affinity between TfR and Fe-transferrin.[3] The role of the H63D mutation on iron overload *in vivo* has been controversial. It has been considered as a simply genetic polymorphism but, recently, in large population studies it has been shown that serum iron indices, particularly transferrin saturation, are higher in subjects with H63D/H63D than in those with the wild type genotype.[4-11] In general all subjects screened for the presence of H63D allele were clinically and/or genetically suspected to have hereditary hemochromatosis, or were relatives of patients with hereditary hemochromatosis. However, the phenotypic expression of this allele, if present, seems to be highly variable and possibly related to other undefined co-inherited genetic modifiers.

HFE gene mutations are frequent in Europe but, whereas C282Y shows a distribution similar to hereditary hemochromatosis, H63D is common even in populations in which the disease has not been reported. The allelic frequency of the H63D

mutation has great variability worldwide, reaching the highest values (>0.20) in Spain.[12,13] In the island of Sardinia, as in other countries not reached by the Celtic influence, the C282Y mutation is very rare (*Melis et al. personal communication*), but H63D shows an allele frequency of 0.173, similar to that found in other populations.[12] In Sardinia there is also a high frequency of β-thalassemia trait (13%) which is characterized by mild, ineffective erythropoiesis that causes an increase of iron absorption.[14] The high frequencies of H63D mutation and of β-thalassemia trait in Sardinians led us to investigate their possible synergistic effect on iron absorption.

Design and Methods

We studied 152 Sardinian adult male heterozygotes for β-thalassemia who came to our service of genetic screening or were selected as parents of thalassemia major patients. Informed consent was obtained from all subjects. We excluded from the study: a) subjects with impaired liver function which could interfere with iron metabolism or ferritin levels; b) females and blood donors, because their iron stores could be reduced as a consequence of menstruation and repeated blood donations; c) children, because iron deposition tends to increase with age and may reach moderately high levels in adults as previously reported;[10] d) subjects with recent and present infections, and liver diseases, which increase ferritin levels. None of the subjects had a history of conditions related to iron deficiency or overload and none had been treated with iron. Red blood cell indices were measured by a Coulter Counter (Gen-S IL, Milan, Italy) and the diagnosis of beta-thalassemia trait was defined on the basis of low mean corpuscular volume (MCV) and mean corpuscular hemoglobin (MCH), and increased HbA2 measured by high performance liquid chromatography (Variant, Bio-Rad, Milan, Italy). Serum ferritin was measured by chemiluminescence (Immulyte, Med System).

DNA analysis

Genomic DNA was extracted from peripheral blood leukocytes by the salting out method.[16] The HFE gene was amplified using the primers and conditions described by Feder *et al.*[1] Polymerase chain reaction (PCR) was performed using a Perkin Elmer Thermal Cycler (Applied Biosystem, Perkin Elmer, Salt Lake City, USA). The PCR products were subsequently digested with the restriction enzymes RsaI (C282Y mutation) and MboI (H63D mutation). Differences between the three groups of subjects were evaluated by the non-parametric Mann-Whitney test.

Table 1. Serum ferritin and hemoglobin values in β-thalassemia heterozygotes according to their H63D genotype.

Genotype H63D	*N° of subjects (%)*	*Ferritin level (μg/L) SD**	*Hemoglobin level (g/dL) ±SD°*
H/H	103 (67.8%)	250 + 138	12.8 + 1.5
H/D	45 (29.5%)	295 + 186	12.7 + 1.1
D/D	4 (2.6%)	389 + 75	13.6 + 0.9

**p values: H/H vs H/D = n.s.; H/D vs D/D = n.s.; H/H vs D/D=0.02;*
°p values: H/H vs H/D = n.s.; H/D vs D/D = n.s.; H/H vs D/D = n.s.

Results

The C282Y mutation was not present in any of the 152 β-thalassemia carriers examined. Forty-five of them were heterozygotes for the H63D mutation (H/D), 4 were D/D homozygotes and 103 were H/H homozygotes. The allelic frequency of H63D mutation was 0.174, similar to that found in the general Sardinian population (*Melis et al., submitted*). Mean serum ferritin levels were calculated in each group and the values are reported in Table 1. There was no difference in age according to the different H63D genotypes. In D/D homozygotes, sequence analysis of the iron responsive element of L-ferritin was performed to exclude a co-inheritance of mutations associated with hyperferritinemia and cataract syndrome,[17] and the results were negative. The hemoglobin concentration (g/dL±SD) is also shown in Table 1. According to the Mann-Whitney test, there is a statistically significant difference between the ferritin values detected in the H/H and D/D groups (p=0.022). No statistically significant difference was detected in ferritin values between the H/D and D/D and between the H/H and H/D groups (Table 1). There was no statistically significant difference in hemoglobin concentration among the three groups.

Discussion

In this study we found higher levels of serum ferritin levels in carriers of β-thalassemia who were homozygotes for H63D mutation than in carriers who were heterozygotes for this HFE allele or who did not have it. Our results confirm the hypothesis that in some way the H63D mutation could be implicated in increasing iron storage when in interaction with other genetic determinants.[18] The comparable hemoglobin levels in the different groups of subjects exclude the possibility that the difference in ferritin levels is due to the degree of anemia which may influence iron absorption. The advantage of our experimental model is that we were

able to select a homogeneous group of subjects with characteristics useful to the understanding of the possible role of H63D allele in iron overload. In fact we studied β-thalassemia carriers who are prone to absorb more iron because of mild anemia and ineffective erythropoiesis.[9]

The correlation between heterozygous β-thalassemia and HFE mutations has been studied by Piperno *et al.*[19] These authors report that β-thalassemia trait may worsen the clinical picture of C282Y homozygotes, favoring higher rates of iron overload, while the association of β-thalassemia trait with heterozygosity for C282Y or H63D alleles does not lead to iron overload. However, the study reports only 2 subjects who were heterozygotes for β-thalassemia and H63D: one had normal iron indices, the other showed increased serum ferritin and transferrin saturation. In our study only the homozygous state for H63D is able to produce statistically significant higher levels of ferritin.

A variable phenotypic presentation of iron overload was found in a large group of H63D homozygotes in whom thalassemia status was not reported.[18] The presence of intragenic or juxtagenic polymorphisms in the HFE gene that could modify the expression of this genotype, influencing the severity of the phenotype, was excluded in these homozygotes and the variable severity of iron overload was attributed to undefined genetic modifiers.[18]

Increased ferritin levels, not associated with iron overload,[17] have been reported in patients with hereditary hyperferritinemia-cataract syndrome (HHCS)[18,19] and have been associated with mutations of the iron responsive element of the L-ferritin gene.[17] These mutations were excluded in our patients with increased ferritin levels.

Even if the level of ferritin increases moderately with age, only a minority of β-thalassemia carriers develop iron overload, and this has been attributed to acquired or genetic factors.[15-20] In some cases iron overload results from inappropriate long-term administration of oral or even parenteral iron, because of diagnostic errors in defining microcytic anemia. Some other cases are due to the presence of severe forms of β-thalassemia. Our subjects are all simple carriers of a unique β-thalassemia mutation, that is the β 39 C→T nonsense mutation, typical of the Sardinian population.[21]

In conclusion, from our results it seems that homozygosity for the H63D allele can be considered a genetic variant that increases the probability of developing iron overload if in association with other genetic determinants such as β-thalassemia.

Contributions and Acknowledgments

MAM and RG conceived the study and drafted the paper; MC analyzed and interpreted the data and revised the literature; FD performed the laboratory tests; SB collected the samples; AC critically reviewed the paper.

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Disclosures

Conflict of interest: none.

Redundant publications: no substantial overlapping with previous papers.

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Peer Review Outcomes

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What is already known on this topic

Previous studies have shown that the HFE mutation C282Y in homozygous states promotes iron overload in β thalassemia trait. The effect of other HFE mutations, such as H63D, in β thalassemia trait has not been systematically investigated.

What this study adds

The study demonstrates that the H63D mutation in the homozygous state results in higher levels of serum ferritin and presumably iron overload in patients with β thalassemia trait. There was no effect on ferritin in the heterozygous state of H63D.

Potential implications for clinical practice

Homozygosity for H63D may promote iron overload in the general population and in β thalassemia trait. A comprehensive assessment of iron metabolism is needed to further elucidate the interplay of H63D and β thalassemia trait.

Carlo Brugnara, Deputy Editor