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The influence of hemochromatosis mutations on iron overload of thalassemia major

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ABSTRACT

Background and Objective. **Hemochromatosis is a genetic form of iron overload due to a defective HFE gene. Secondary iron overload is the main complication in transfusion-dependent thalassemia patients. In this work we have examined the prevalence of HFE mutations in thalassemia major and evaluated the degree of iron overload of patients with and without HFE mutations.**

Design and Methods. **HFE mutations were studied in 71 Italian thalassemic patients and in 189 normal controls, using PCR and restriction enzyme analysis. The degree of iron overload, assessed by serum ferritin and liver iron concentration (LIC), was compared in 17 patients with mutations in the HFE gene, and in 17 subjects with wild type HFE genotype. The two groups of patients had comparable globin gene mutations, were matched for age and were homogeneous for transfusion and chelation history. In all cases the *iron balance* calculated on the basis of transfusion regimen and iron excreted by chelation was available.**

Results. **The allele frequencies of C282Y and H63D were respectively 1.4% and 12.7% in patients and 1.1% and 11.4% in controls. No case of C282Y homozygosity was recorded among patients. No significant difference was found in terms of serum ferritin, LIC, or the age at chelation start between patients with and without HFE mutations. The single patient with H63D homozygosity was severely iron-loaded.**

Interpretation and Conclusions. **Our data suggest that the presence of a single mutation in the HFE gene does not influence the severity of iron loading in thalassemia patients following a regular transfusion and chelation program.**
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Key words: iron, iron overload, thalassemia, HFE mutations, liver iron, iron balance

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In Mediterranean countries the most common cause of iron overload is homozygous β-thalassemia. Iron accumulation in thalassemic patients depends both on increased intestinal iron absorption – which is proportional to the degree of erythroid hyperplasia – and on blood transfusions. Progress in iron-chelating therapy over the last twenty years has dramatically changed the prognosis of these patients, as iron overload may be maintained at low levels in regularly transfused thalassemic subjects by applying lifelong regular chelation.[1]

Primary iron overload among Caucasians is caused by hereditary hemocromatosis (HH), a common autosomal recessive disorder characterized by an inappropriately high intestinal iron absorption. Two mutations have been identified in HFE, the hemochromatosis gene. A missense mutation causes a cysteine to tyrosine substitution at position 282 of the protein (C282Y) and the disruption of the β_2 microglobulin binding site.[2] A second mutation changes a histidine residue at codon 63 to aspartic acid (H63D). C282Y is present in the homozygous state in most HH patients so far studied.[2-6] The role of H63D in the disease is controversial. The H63D protein, in contrast to C282Y, is normally expressed on cell surface[7] and the risk of iron overload in H63D homozygotes appears to be only modestly (about four fold) increased.[8] However, recent data suggest a causal role for this mutation. According to *in vitro* observations H63D mutation results in a decreased inhibition of TfR affinity for diferric transferrin.[9,10] If these results are confirmed in vivo it could be hypothesized that both mutations may modify the iron loading in thalassemia. In agreement with this hypothesis we have observed a significant degree of iron overload in a small number of patients with heterozygous β-thalassemia and a mutation (either C282Y or H63D) in the HFE gene.[11]

In this work we investigated whether HFE mutations modulate iron overload of thalassemia major. First, we examined the incidence of C282Y and H63D mutations among Italian patients with homozygous β-thalassemia. Next, we accurately evaluated the

degree of iron loading in selected patients, matched for age and homogeneous for transfusion-chelation history, classified into two groups according to the HFE genotype.

Design and Methods

Patients studied

Seventy-one unselected, unrelated, transfusion-dependent β-thalassemic patients followed at the Pediatric Department of the University of Turin were included in the genetic study. There were 40 females and 31 males, aged 1-27 years (mean age 19 years). All patients were treated according to a computerized transfusion scheme to mantain the pretransfusional hemoglobin (Hb) level above 95 g/L and the mean Hb at 120 g/L by administering filtered red blood cell concentrates. Desferrioxamine (30-50 mg/kg/daily) was administered by subcutaneous infusions over 8-10 hours 5-7 days/week. Iron chelation was usually started after transfusions of 10-20 units of blood or when serum ferritin approximated 1,000 μg/L.[12] The mean compliance with chelation was 0.80.[13] All these patients were studied for HFE and β-globin mutations. Thirty-five patients were eligible for detailed evaluation of iron overload.

Controls were unrelated blood donors with normal iron parameters. Both patients and controls were Italian, living in Piedmont, a region where the population is of mixed origin, both from Northern and Southern Italy.

Informed consent was obtained for the study from the patients or their parents, according to the guidelines of the local institutions.

Molecular studies

DNA was obtained from peripheral blood buffy coats by standard methods.[14] PCR was performed in a thermal cycler (Perkin Elmer) using 12.5 pmoles of each primer and 0.5 U of Taq DNA polymerase, for a total of 30 cycles.

β-globin gene mutations were assessed by PCR and reverse dot blot[15] and/or the allele-refractory mutation system (ARMS).[16]

C282Y and H63D mutations were identified by restriction enzyme analysis of PCR-amplified DNA, as described elsewhere.[3] In brief, aliquots of the PCR products were digested with Rsa I and Mbo I restriction enzymes (New England Biolabs, Berkeley, MA, USA), in order to identify respectively C282Y and H63D variants. Digestion conditions were those recommended in the manufacturer's protocols. After digestion, fragments were electrophoresed on 2% agarose gels.

Evaluation of iron overload

The degree of iron overload was evaluated by multiple parameters:

i) liver (LIC) and spleen (SIC) iron concentrations were measured using a SQUID biosusceptometer (Ferritometer®, BTi, USA) as described elsewhere;[17,18]

ii) serum ferritin was determined at the time of SQUID analysis; the mean serum ferritin of the previous 3 years was also considered (on the average of 5 determinations yearly). Ferritin was determined by fluoroimmunoassay (Autodelfia, Wallac Oy, Finland);

iii) iron balance was assessed calculating the difference between transfusional iron input and chelation-induced iron output. Calculations were made according to accepted International Guidelines of the *Management Protocol for the Treatment of Thalassemia Patients* (1997 edition) in cooperation with W.H.O. and distributed by the *Thalassemia International Federation* (Nicosia, Cyprus). Iron balance was determined in two subgroups of patients (see below) for whom clinical records were available from the first year of life.

Iron input was calculated from the net weight of blood transfused and hematocrit. Iron output was calculated from the monthly determination of 24 hour urinary iron excretion, adding a 30% fecal excretion, multiplied for the number of recorded desferrioxamine infusions.

Statistics

The comparison between groups was performed by Student's t test using Statistica 5.0 software (StatSoft, Inc., Tulsa, OK, USA).

Results

Results of HFE mutation analysis are reported in Table 1. As expected, the frequency of C282Y and H63D was similar in patients and controls. Eighteen patients had HFE mutations. One patient was heterozygous for C282Y, one was compound heterozygote for C282Y and H63D and 15 had H63D at the heterozygote state. A single patient was H63D homozygote.

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Table 1. HFE mutations in β-thalassemia major.

HFE genotype	*Patients* *n*	*%*	*Controls* *n*	*%*
C282Y/N	1	1.4	4	2
C282Y/C282Y	0	-	0	-
C282Y/H63D	1	1.4	0	-
H63D/N	15	21	39	20
HD63D/H63D	1	1.4	2	1
N/N	53	74.6	144	76
Total	71	189		
Allele frequency				
C282Y	1.4	1.1		
H63D	12.7	11.4		

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patients with wild type HFE genotype, matched for age, severity of thalassemia mutations and treatment history are shown in Table 2. The age at start of chelation and the calculated iron balance was similar among patients with and without HFE mutations. Although the values of iron balance are rather widely spread, this distribution – predominantly caused by individual variations in iron excretion – was observed in both groups. The degree of iron overload, expressed in terms of serum ferritin at the time of SQUID, mean ferritin and LIC, was not statistically different in the two groups. Measurement of iron stores in our patients was done non-invasively by SQUID because of the young age of the subjects, taking into consideration the accuracy of the method in comparison to the biochemical assay, which requires liver biopsy.[18]

The single homozygous H63D patient had unusually severe iron overload. This subject was a 6-year old female with β°/Lepore thalassemia, regularly transfused from the age of 8 months. Sequential evaluations showed extremely high levels of both serum ferritin and LIC (3,462 µg/L and 5,011 µg/mg at 3.8 years of age; 4,425 µg/L and 5,392 µg/mg at 6.8 years respectively), in spite of an unchanged iron balance and of a regular chelation. Her LIC values were the highest in our series and there was a significant discrepancy between LIC and the calculated iron-balance. Liver biopsy performed in view of bone mar-

Table 2. Clinical data and iron parameters of the patients studied.

Pt. n.	*Age yrs*	*Gender*	*β-globin genotype mutations*	*HFE genotpe C282Y*	*H63D*	*Age at start chelation yrs.*	*Iron input mg*	*Iron output mg*	*Iron balance mg*	*SF at SQUID µg/L*	*Mean SF µg/L*	*LIC µg/g/liver wet weight*
1	14.6	M	CD 39/ IVSI:6	+/-	+/-	1.2	79,339	61,623	17,716	3,207	1,493	2,140
2	17.5	F	CD 39/ CD39	+/-	-/-	3.5	73,139	57,846	15,293	1,163	976	922
3	8.1	F	IVSI:110/ IVSI:I	-/-	+/-	3.7	27,801	23,400	4,401	1,678	1,369	894
4	11.5	F	CD 39/ IVSII:1	-/-	+/-	1.5	57,729	37,090	20,639	1,354	963	1,230
5	12.1	M	IVSI:1/ IVSII:745	-/-	+/-	2.4	63,881	49,400	14,481	1,344	1,182	600
6	14.9	M	CD 39/ IVSI:6	-/-	+/-	4.3	61,295	65,676	-4,381	1,862	1,616	679
7	16.0	F	CD 39/ IVSI:6	-/-	+/-	2.6	89,752	67,786	21,966	1,352	1,247	830
8	16.2	F	CD 39/ IVSI:110	-/-	+/-	3.7	93,519	78,000	15,519	1,027	767	860
9	17.6	M	CD 39/ CD 39	-/-	+/-	3.6	98,739	61,194	37,545	1,441	879	1,450
10	18.2	M	IVSI:110/ IVSI:110	-/-	+/-	4.6	105,798	59,355	46,442	3,209	1,608	1,700
11	18.7	M	CD 39/ CD39	-/-	+/-	4.7	96,939	68,900	28,039	2,091	1,305	1,900
12	19.5	M	IVSI:110/ IVSI:6	-/-	+/-	3.6	117,669	78,000	39,669	2,305	1,641	2,200
13	19.7	F	IVSI:1/ IVSI:1	-/-	+/-	5.0	96,701	69,742	26,959	1,587	802	1,990
14	19.9	M	CD 39/ IVSI:6	-/-	+/-	4.1	147,669	131,300	16,369	1,583	2,240	484
15	20.9	M	IVSI:110/ IVSI:110	-/-	+/-	5.9	110,895	81,900	28,995	1,646	903	2,537
16	21.1	M	IVSI:110/ IVSI:6	-/-	+/-	5.2	118,569	72,937	45,632	4,000	3,240	885
17	21.4	M	IVSI:110/ IVSI:6	-/-	+/-	10.5	86,281	78,000	8,281	825	971	690
mean	17.1					4.4	89,748	67,185	22,563	1,863	1,365	1,294
St. dev.	3.9					2.1	28,065	22,469	14,150	862	619	656
18	15.1	M	CD 39/ CD 39	-/-	-/-	2.7	83,297	47,085	36,212	1,861	1,310	2,310
19	7.2	M	CD 39/ IVSI:6	-/-	-/-	2.9	21,409	8,918	12,491	1,838	1,915	1,010
20	9.9	M	CD 39/ CD 39	-/-	-/-	2.0	37,733	13,289	24,445	2,453	2,604	1,721
21	13.1	M	CD 39/ CD 39	-/-	-/-	1.0	54,628	33,417	21,211	1,694	1,264	1,150
22	14.1	M	CD 39/ IVSI:6	-/-	-/-	2.6	58,861	33,775	25,086	1,925	1,891	1,210
23	18.0	F	IVSI:110/ IVSI:1	-/-	-/-	3.9	103,015	80,253	22,762	745	747	1,267
24	16.8	F	IVSI:110/ IVSI:110	-/-	-/-	3.3	109,428	65,441	43,987	1,879	1,731	2,100
25	17.6	M	CD 39/ CD 39	-/-	-/-	4.1	86,374	41,012	45,362	2,508	1,944	1,740
26	18.9	M	IVSI:1/ IVSI:6	-/-	-/-	5.3	127,177	122,200	4,977	793	953	530
27	14.7	M	CD 39/ CD 39	-/-	-/-	2.3	79,818	52,571	27,248	1,961	1,272	1,260
28	19.3	M	CD 39/ CD 39	-/-	-/-	4.8	118,522	89,317	29,205	2,651	2,474	2,590
29	21.3	F	CD 39/ CD 39	-/-	-/-	5.7	121,206	100,998	20,208	2,238	1,579	1,195
30	20.2	M	IVSI:110/ IVSI:1	-/-	-/-	6.3	43,386	50,700	-7,314	738	987	210
31	20.5	M	CD 39/ IVSI:110	-/-	-/-	6.5	130,233	117,000	13,233	2,619	1,704	648
32	20.3	F	IVSI:6/ IVSII:745	-/-	-/-	5.3	126,073	119,600	6,473	642	758	474
33	19.7	F	CD 39/ CD 39	-/-	-/-	5.1	131,338	113,100	18,238	1,999	1,053	1,637
34	18.8	F	IVSI:110/ IVSI:110	-/-	-/-	5.3	74,984	49,400	25,584	863	723	1,128
mean	16.8					4.1	88,675	66,946	21,730	1,730	1,465	1,305
St. dev.	4.0					1.6	35,823	37,597	13,495	707	581	655

SF = serum ferritin; LIC = liver iron concentration.

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row transplantation revealed advanced fibrosis and heavy iron deposition within both hepatocytes and Kupffer cells.

Discussion

The presence of HFE mutations may adversely affect the course of a number of disorders, including porphyria cutanea tarda,[19] sideroblastic anemia[20] and β-thalassemia intermedia.[21,22] Data on the interaction of primary hemochromatosis in thalassemia patients are scanty. Severe iron overload was reported in a 61-year old Indian patient with thalassemia intermedia, heterozygous for C282Y.[21] These findings allowed speculation that defective HFE might behave as a modifying gene also in thalassemia major. On the other hand, it has recently been shown that HFE mutations do not modify the clinical picture of regularly transfused and chelated thalassemia major patients, suggesting that optimal medical treatment is able to overcome the potential effect on iron absorption caused by the defective HFE gene.[23] In the latter series no significant differences in serum ferritin measurements, LIC or development of iron-induced complications were found in C282Y heterozygotes and in carriers of H63D both at heterozygous and homozygous states, as compared to patients without HFE mutations, although the highest mean values of LIC and serum ferritin were found among carriers of H63D.[23]

Comparison of the severity of iron overload among transfused thalassemia patients is a complex issue. Several factors influence the iron burden in these subjects, including the transfusion protocol, the iron chelation regimen, the patient's compliance to treatment and the β-globin molecular defect. In order to evaluate the role of increased iron absorption due to HFE mutations we tried to minimize the effect of all the other factors causing iron overload. There was no remarkable difference in the β-globin genotype in patients with and without HFE mutations, although the effect of β-globin genotype – which indirectly influences iron absorption through the severity of chain imbalance and the degree of anemia – is of limited relevance in patients who have been regularly transfused since diagnosis. The effect of blood transfusions is minimized in patients treated with a homogeneous protocol, and can be quantified when clinical records are available.[12] By analogy, the effect of iron chelation (age at start, dose of chelators and especially compliance to treatment) may be assessed in each patient. Quantification of iron transfused and removed can be used to calculate the iron balance, which represents the estimate of treatment-dependent iron load. As shown in Table 2 this value was comparable in the two groups of patients.

No difference was observed in terms of serum ferritin and LIC in the two groups, selected on the basis of comparable iron balance. Although the series examined is small, the results obtained suggest that iron overload in thalassemia patients is not influenced by the HFE genotype. In agreement with a previous report, we conclude that the treatment protocol is able to overcome the effect on iron absorption that may be caused by a defective HFE gene.[23] The lack of difference in age at starting chelation suggests that the suppression of the role of the defective HFE gene, if any, is transfusion-dependent.

In the single patient homozygous for H63D, serum ferritin and LIC were disproportionate to the patient's age, and significantly higher than expected on the basis of the iron balance. In addition, advanced fibrosis and heavy iron deposition are unusual liver biopsy findings at this age in a treated patient. Since the patient has a HLA identical-sister, bone marrow transplantation was planned. The potential donor has two chromosome 6p haplotypes identical to the proband and is H63D homozygote as well. Bone marrow transplantation, if successful, will correct the more severe of the two inherited disorders; nevertheless, iron overload is well amenable to venesections in transplanted β-thalassemic patients.[24] No other H63D homozygotes were present in our series and no similar observations exist in the literature. Since other H63D homozygotes in thalassemia major do not have severe iron overload (*C. Borgna-Pignatti, R. Galanello, personal communications*), studies are in progress to assess whether other iron-defective genes are interacting in the unusual case presented.[25]

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CC and AP were responsible for designing the study and writing the paper. FL was responsible for clinical data analysis. GZ and LS were responsible for molecular studies and their interpretation. RF provided the SQUID facility and data intepretation. All the authors gave a critical contribution to the paper and approved the final version. The name order was a joint decision, considering the role of each author.

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Disclosures

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