Role of iron metabolism genetic determinants in response to chelation therapy in a cohort of β -thalassemia and sickle cell syndromes Italian patients

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Abstract

In patients with β -thalassemia and sickle cell syndromes there is an important secondary iron overload due to regular blood transfusions and increased duodenal iron absorption. As in genetic hemochromatosis, also the secondary iron storage leads to tissue injury that involves all the major organs: liver, heart, kidney, endocrine glands. At present, in patients with β -thalassemia and sickle cell syndrome, iron chelation therapy is widely used for the treatment of secondary hemochromatosis, to limit the toxic effects of iron overload. In order to maintain the correct homeostasis, several genes are involved in the metabolic pathways of iron, including *HFE*, *FPN* (ferroportin) and *TF* (transferrin). In this study we analyzed the genes *HFE*, *FPN* and *TF*, to assess their possible effects on response to therapy with deferasirox and deferiprone, either as monotherapy or in combination therapy in a cohort of patients with β -thalassemia and sickle cell syndromes.

Introduction and Rationale

In patients with β -thalassemia and sickle cell syndrome, in addition to anemia due to a deficient production of β -globin chains, there is significant iron storage in the main organs due to periodic blood transfusions, and to an increased absorption of iron in the duodenum approximately three times higher than the normal. As in genetic hemochromatosis,^{1,3} also the secondary iron storage leads to tissue injury that involves all the major organs: liver, heart, kidney, endocrine glands.

Iron storage, especially when secondary to a non-intensive blood transfusion regimen, may be worsened by mutations in genes responsi-

ble for primary hemochromatosis, and by an inappropriate mechanism of hepcidin regulation.^{4,6}

The presence of one or both of such conditions may give rise, in these patients, to variations in the severity of secondary hemochromatosis and response to chelation therapy.

In order to maintain the correct homeostasis, several genes are involved in the metabolic pathways of iron, including *HFE* (high iron Fe), *FPN* (ferroportin) and *TF* (transferrin).

The *HFE* gene is responsible for hemochromatosis type I and it is involved in the regulation of iron-transferrin binding. Mutations in *HFE* cause failure of hepcidin synthesis and intrahepatic iron accumulation.⁷

The *FPN* gene encodes for the protein ferroportin (FP). FP is the main cellular iron exporter, it is regulated by hepcidin and promotes the iron release from enterocytes and macrophages towards the bloodstream. Mutations within *FPN* lead to an alteration of FP the functions and are responsible for autosomal dominant hereditary hemochromatosis Type IV, also called *ferroportin disease*, characterized by iron stores in the reticulum endothelium.^{8,9}

The most common *FPN* mutations are *loss of function*, they reduce FP expression causing iron retention and restrict iron available for erythropoiesis.^{8,10}

Moreover, there are very rare *FPN* gain-of-function mutations making FP resistant to hepcidin regulation. These FP variants give rise to a hepcidin-resistant hemochromatosis, where FP is not internalized and remains on the macrophages surface, increasing the share of available iron.¹¹

TF gene encodes for transferrin, a glycoprotein whose function is the transport of iron from the intestine, reticuloendothelial system, and liver parenchymal cells to all proliferating cells in the body. *HFE* and *TF* compete for binding the *TF* receptor.^{7,9}

Mutations in *TF* are responsible for familial hypotransferrinemia, a rare recessive disease characterized by an extremely low transferrin level [0.1 g/L (10 mg/dL)] and severe hypochromic anemia with iron overload in the liver and other parenchymal organs. Mutations causing the total absence of *TF* are incompatible with life.⁷⁻⁹

At present, in patients with β -thalassemia and sickle cell syndrome, iron chelation therapy is widely used for the treatment of secondary hemochromatosis to limit the toxic effects of iron overload.

So far, different chelators are used in protocols of iron chelation in polytransfused patients. Deferasirox (DFX) and deferiprone (DFP) are two of the most widely used chelators, having different ways of iron binding.¹²

DFX is a tridentate orally administered iron chelator. DFX is rapidly absorbed, and the main

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route of excretion is in the feces.¹²

DFP chemically behaves as an antioxidant; it is a molecule able to bind the iron in a molar ratio of 3:1, absorption is rapid and the drug is mainly excreted in the urine.¹²

In this study we analyzed the genes *HFE*, *FPN* and *TF*, to assess the possible effects on response to therapy with DFX and DFP, either as monotherapy or in combination therapy in a cohort of patients with β -thalassemia and sickle syndromes.¹³ To this aim different levels of serum ferritin have been analyzed and compared in patients undergone to the same therapeutic chelating protocol but with different genotypes.

Materials and Methods

Thirty patients, aged between 16 and 65 years, were studied: twenty-nine were affected by β -thalassemia and 1 was affected by sickle cell syndrome.

β -thalassemia patients were regularly transfused, while the patient with sickle cell syndrome was not transfused. The demographic and clinical characteristics of all patients are summarized in Table 1.

Patients were divided into 3 treatment groups: i) DFX-monotherapy; ii) DFP-

monotherapy; iii) sequential therapy- DFX 3 d/w - DFP 4 d/w (Table 1).

Having obtained the informed consent for molecular studies according to institutional guidelines, genomic DNA was extracted from peripheral blood mononuclear cells by means of sodium dodecyl sulfate-phenol/chloroform extraction technique.

HFE, *FPN* and *TF* genes encoding and flanking regions were investigated by direct genomic sequencing and restriction endonucleases. Specific polymerase chain reactions (PCR) and direct sequencing primers for *HFE*, *FPN* and *TF* genes were designed in our laboratory on the basis of sequence databases (GENATLAS: <http://www.ncbi.nlm.nih.gov>).

Specific PCRs were carried out on genomic DNAs in a thermal cycler using 10 mM each primers and 2.5 U Taq polymerase in a final volume of 50 μ L. Direct sequencing was performed using a Genome Lab DTCS Quick Start Kit for Dye Terminator Cycle Sequencing (Beckman Coulter, Inc., Brea, CA, USA), in the Genetic Analysis System CEQ8800 (Beckman Coulter) (Figure 1).

The S208S polymorphism in the *TF* gene was investigated by the restriction endonuclease Dde I (Figure 2).

patients were divided into three groups depending on chelation therapy they underwent. For each group of patients, *HFE*, *FPN* and *TF* genotypes were related to specific chelation therapy, the number of transfusions per year and the mean values of ferritin.

HFE gene

Of the 30 patients analyzed, 10 (33.3%) were heterozygous for the H63D mutation, 1 (3.3%) was heterozygous for the C282Y mutation; none of the patients was homozygous or compound heterozygous (Table 2).

The allele frequencies of the C282Y and H63D variants were 16.7% and 1.7%, respectively (Sicilian healthy people allele frequency 20.5% and 0.7%).

FPN gene

None of the analyzed patients had mutations in the *FPN* 3,5,6,7,8 exons. Fourteen out of thirty (46.6%) were homozygous for the not-disease linked polymorphism Val221Val T>C; 12/30 (40%) were heterozygous and 4/30 (13.4%) were negative for this polymorphism (Table 2).

TF gene

For the *TF* gene were analyzed the polymorphism S208S, and the variant G277S, the latter in linkage disequilibrium with the polymorphism L247L. These variants are all associated with a reduction of total iron binding capacity.

Seventeen out of thirty patients (56.7%) were negative for all the analyzed variants; 8/30 (26.7%) patients were heterozygous for the polymorphism S208S; 1/30 (3.3%) was homozygous S208S; 1/30 (3.3%) was heterozygous for the polymorphism L247L; 3/30 (10%)

Table 1. Demographic and clinical features of the 30 studied patients.

	No.
Gender	
Males	9
Females	21
Mean age (years)	34.23 (SD $\pm$ 11.92)
Hemoglobinopathies	
β -thalassemia	29
Sickle cell disease	1
Transfusional therapy	
Yes	29
No	1 (patient with SCD)
Iron chelation therapy	
Yes	30
No	0
DFX monotherapy	13
DFP monotherapy	11
Sequential therapy DFX 3 d/w DFP 4 d/w	6

SD, standard deviation; SCD, sickle cell disease; DFX, deferasirox; DFP, deferiprone.

Results

Results are summarized in Table 2. The

Figure 1. Direct gene sequencing. A) exon 2 of *HFE* gene: heterozygous H63D; B) exon 7 of *TF* gene: heterozygous L247L; C) exon 7 of *TF* gene: heterozygous G277S.

1 2 3 4 5 6 7 8 9 10 11 12 13 14

Figure 2. TF S208S polymorphism Dde I restriction fragments. Lanes 1 and 14: ϕ X174 Hae III; lane 2 negative control; lanes 4, 5, 7, 8 and 12: negative samples; lanes 3, 6, 9, 11 and 13: heterozygous samples; lane 10: homozygous sample.

were heterozygous for both the polymorphism L247L and the mutation G277S (Table 2).

Discussion and Conclusions

In Sicilian population one of the major causes of iron overload is the state of homozygosity for β -thalassemia and sickle cell syndrome. In these patients, iron storage is due to blood regular transfusions, and to the increase of iron absorption in the duodenum secondary to erythroid hyperplasia.

Iron-chelating therapy allows controlling iron storage.

In these patients the presence of mutations responsible of hereditary hemochromatosis, could influence the severity of secondary hemochromatosis and the response to chelation therapy.

All patients but one, of the present study were regularly transfused and all underwent chelation therapy: DFX-monotherapy, DFP-monotherapy or 3-sequential therapy DFX 3 d/w- DFP 4 d/w.

Because none of the patients showed mutations within the gene *FPN*, for this cohort of patients is not possible to determine the influence of the *FPN* gene on the response to iron chelation therapy.

Data obtained by molecular analysis of the

HFE and *TF* genes, suggest that in β -thalassemia patients regularly transfused, the heterozygous state for one of three major *HFE* mutations (C282Y, H63D, S65C) and/or the presence of *TF* gene variants, does not affect the serum ferritin levels.

In fact, the serum ferritin levels found in these patients, do not differ with regard to different *HFE* and *TF* genotypes, but to the number of transfusions per year and to the chelation therapy they undergo. This indicating the transfusional protocol and the chelation regimen as the main indicators of the severity and variability of iron overload.

Table 2. Patients' genotypes and phenotypes related to transfusional and chelating therapy.

	Gender	Age	Phen. β-thal	Genotypes						TX/Y	Ferritin ng/mL
				β-globin	HFE	FPN	S208S	TF L247L	G277S		
Group 1 DFX											
1	F	23	TM	Cd39/IVS1,nt110	H63D/N	N/N	Het	N	N	16	587.1
2	F	51	TI	Cd39/IVS1,nt6	H63D/N	N/N	N	N	N	12	5.680
3	M	36	TM	IVS1 nt110/IVS1,nt1	N/N	N/N	Het	N	N	26	2.079
4	M	31	TI	IVS1,nt 6/ IVS1,nt 110	H63D/N	N/N	N	N	N	20	799.8
5	F	43	TM	Cd39/Cd39	N/N	N/N	Het	N	N	18	471.9
6	M	35	TM	IVS1,nt110/IVS1, nt1	N/N	N/N	N	N	N	16	1.838
7	F	36	TM	IVS1,nt110/IVS1,nt110	N/N	N/N	Het	N	N	38	2.243
8	F	39	TM	IVS1,nt1/IVS1,nt5	H63D/N	N/N	N	N	N	17	1476
9	F	20	TM	Cd39/IVS1,nt110	H63D/N	N/N	N	N	N	28	1871
10	F	37	TM	Cd39/Cd39	H63D/N	N/N	N	N	N	27	1891
11	F	50	TM	IVS1,nt6/ fr.76	N/N	N/N	N	Het	Het	19	824.6
12	F	56	TI	IVS1,nt10/IVS1,nt6	N/N	N/N	N	N	N	15	417.2
13	M	30	TM	IVS1,nt1/ IVS1,nt6	H63D/N	N/N	N	Het	Het	12	1.806
Group 2 DFP											
1	F	65	TI	IVS2, nt1/NI	H63D/N	N/N	N	N	N	13	2.036
2	F	25	TM	INS2, nt1/ IVS1, nt110	H63D/N	N/N	N	N	N	20	928
3	F	31	TM	IVS1,nt110/IVS1,nt110	C282Y/N	N/N	Homoz	N	N	24	1.415
4	M	34	TM	Cd39/Cd39	N/N	N/N	N	N	N	13	1690
5	F	53	TM	Cd39/IVS1,nt110	N/N	N/N	Het	N	N	19	837.5
6	F	33	TM	IVS1,nt110/IVS1,nt110	N/N	N/N	N	N	N	21	1.532
7	F	23	TM	Cd39/IVS1,nt110	H63D/N	N/N	N	N	N	27	1.652
8	M	16	TM	Cd39/IVS1,nt110	N/N	N/N	Het	Het	N	21	1.611
9	F	20	TI	IVS1,nt6/IVS1,nt110	N/N	N/N	N	N	N	19	1.888
10	M	32	TI	IVS1,nt6 /IVS1,nt 6	N/N	N/N	Het	N	N	14	676.2
11	F	27	SCD	βS/βS	N/N	N/N	N	N	N	0	896.6
Group 3 DFX 3 d/w DFP 4 d/w											
1	F	32	TM	Cd39/Cd39	N/N	N/N	N	N	N	22	2.915
2	M	21	TM	IVS1,6/Cd39	N/N	N/N	N	Het	Het	13	1.547
3	M	31	TM	Cd39/IVS1nt110	N/N	N/N	N	N	N	23	1.696
4	F	43	TM	Cd39/-87	N/N	N/N	N	N	N	17	1027.3
5	F	36	TM	Cd39/IVS1,nt6	N/N	N/N	Het	N	N	13	1200
6	F	18	TM	Cd39/IVS1,nt6	N/N	N/N	Het	N	N	16	2.877

Phen., phenotype; DFX, deferasirox; TM, thalassemia major; N, negative; Het, heterozygous; TI, thalassemia intermedia; DFP, deferiprone; Homoz, homozygous; SCD, sickle cell disease.

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