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, 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-
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 primer and 2.5 U Taq polymerase in a final volume of 50 µL. 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).

**Results**

Results are summarized in Table 2. The 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%) was homozygous S208S.

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

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

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

![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.](image)

![Figure 2. TF S208S polymorphism Dde I restriction fragments. Lanes 1 and 14: ϕX174 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.](image)
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 

| Gender | Age | Phen. | β-globin | HFE | FPN | S208S | TF | L247L | G277S | TX/Y | Ferritin ng/mL |
|--------|-----|-------|----------|-----|-----|-------|----|-------|-------|------|----------------|
| 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   | 5860.0         |
| 3      | M   | 36    | TM       | IVS1 nt110/IVS1,nt1 | N/N   | N/N   | Het | N     | N     | 26   | 2079.9         |
| 4      | M   | 31    | TI       | IVS1,nt6/IVS1,nt10 | H63D/N | N/N   | N   | N     | N     | 20   | 798.8          |
| 5      | F   | 43    | TM       | G277S       | 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   | 1838.8         |
| 7      | F   | 36    | TM       | IVS1,nt110/IVS1,nt10 | N/N   | N/N   | Het | N     | N     | 38   | 2243.3         |
| 8      | F   | 39    | TM       | IVS1,nt1/IVS1,nt5 | H63D/N | N/N   | N   | N     | N     | 17   | 1476.0         |
| 9      | F   | 20    | TM       | Cd39/IVS1,nt110 | H63D/N | N/N   | N   | N     | N     | 28   | 1871.2         |
| 10     | F   | 37    | TM       | Cd39/IVS1,nt9 | H63D/N | N/N   | N   | N     | N     | 27   | 1891.0         |
| 11     | F   | 50    | TM       | IVS1,nt6/IVS1,nt7 | N/N   | N/N   | Het | Het   | N     | 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   | Het | Het   | N     | 12   | 1806.0         |

**Group 2 DFP**

| Gender | Age | Phen. | β-globin | HFE | FPN | S208S | TF | L247L | G277S | TX/Y | Ferritin ng/mL |
|--------|-----|-------|----------|-----|-----|-------|----|-------|-------|------|----------------|
| 1      | F   | 65    | TI       | IVS2, nt1/N   | H63D/N | N/N   | N   | N     | N     | 13   | 2036.0         |
| 2      | F   | 25    | TM       | INS2, nt1/IVS1,nt110 | H63D/N | N/N   | N   | N     | N     | 20   | 928.8          |
| 3      | F   | 31    | TM       | IVS1,nt110/IVS1,nt10 | C282Y/N | N/N   | Homo | N   | N     | N     | 24   | 1415.0         |
| 4      | M   | 34    | TM       | Cd39/IVS1,nt9 | N/N   | N/N   | N   | N     | N     | 13   | 1690.0         |
| 5      | F   | 53    | TM       | IVS1,nt1/IVS1,nt10 | N/N   | N/N   | Het | N     | N     | 19   | 837.5          |
| 6      | M   | 33    | TM       | IVS1,nt110/IVS1,nt10 | N/N   | N/N   | N   | N     | N     | 21   | 1532.0         |
| 7      | F   | 23    | TM       | Cd39/IVS1,nt110 | H63D/N | N/N   | N   | N     | N     | 27   | 1652.2         |
| 8      | M   | 16    | TM       | Cd39/IVS1,nt10 | N/N   | N/N   | Het | Het   | N     | 21   | 1611.1         |
| 9      | F   | 20    | TI       | IVS1,nt6/IVS1,nt110 | N/N   | N/N   | N   | N     | N     | 19   | 1888.0         |
| 10     | M   | 32    | TI       | IVS1,nt6/IVS1,nt6 | N/N   | N/N   | Het | N     | N     | 14   | 676.2          |
| 11     | F   | 27    | SCD     | βSβS       | N/N   | N/N   | N   | N     | N     | 0    | 896.6          |

**Group 3 DFX 3 d/w DFP 4 d/w**

| Gender | Age | Phen. | β-globin | HFE | FPN | S208S | TF | L247L | G277S | TX/Y | Ferritin ng/mL |
|--------|-----|-------|----------|-----|-----|-------|----|-------|-------|------|----------------|
| 1      | F   | 32    | TM       | Cd39/IVS1,nt10 | N/N   | N/N   | N   | N     | N     | 22   | 2915.3         |
| 2      | M   | 21    | TM       | IVS1,nt6/IVS1,nt9 | N/N   | N/N   | N   | Het   | N     | 13   | 1547.1         |
| 3      | M   | 31    | TM       | Cd39/IVS1,nt110 | N/N   | N/N   | N   | N     | N     | 23   | 1696.0         |
| 4      | F   | 43    | TM       | Cd39/IVS1,nt10 | N/N   | N/N   | N   | N     | N     | 17   | 1027.3         |
| 5      | F   | 36    | TM       | Cd39/IVS1,nt6  | N/N   | N/N   | N   | N     | N     | 13   | 1200.0         |
| 6      | F   | 18    | TM       | Cd39/IVS1,nt6  | N/N   | N/N   | N   | N     | N     | 16   | 2877.3         |

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

1. Fiorelli G, Fargion S, Piperno A, et al. Iron metabolism in thalassemia intermediate. Haematologica 1990;75:89-95.
2. Allen KJ, Gurrin LC, Constantine CC, et al. Iron-overload–related disease in HFE hereditary hemochromatosis. N Engl J Med 2008;358:221-30.
3. Pietrangelo A. Hereditary hemochromatosis - a new look at an old disease. N Engl J Med 2004;350:2383-97.
4. Cianetti L, Gabbianelli M, Sposi NM. Ferroportin and erythroid cells: an update. Adv Hematol 2010;2010. pii: 404173.
5. Pietrangelo A. Hereditary hemochromatosis. Biochim Biophys Acta 2006;1763:700-10.
6. Politou M, Kalotychou V, Pissia M, et al. The impact of the mutations of the HFE gene and of the SLC11A3 gene on iron overload in Greek thalassemia intermedia and bS/bthal anemia patients. Haematologica 2004;89:490-2.
7. Pietrangelo A. Hemochromatosis: an endocrine liver disease. Hepatology 2007;46:1291-301.
8. Ganz T, Nemeth E. The hepcidin-ferroportin system as a therapeutic target in anemias and iron overload disorders. Hematology Am Soc Hematol Educ Program 2011;2011:538-42.
9. Pietrangelo A. Molecular insights into the pathogenesis of hereditary haemochromatosis. Gut 2006;55:564-8.
10. Mayr R, Janecke AR, Schranz M, et al. Ferroportin disease: a systematic meta-analysis of clinical and molecular findings. J Hepatol 2010;53:941-9.
11. Camaschella C Silvestri L. Molecular mechanisms regulating hepcidin revealed by hepcidin disorders. Sci World J 2011;11:1357-66.
12. Maggio A. Light and shadows in the iron chelation treatment of haematological diseases. Br J Haematol 2007;138:407-21.
13. Maggio A, Vitrano A, Capra M, et al. Long-term sequential deferiprone-deferoxamine versus deferiprone alone for thalassemia major patients: a randomized clinical trial. Br J Haematol 2009;145:245-54.