

### Autosomal dominant reticuloendothelial iron overload (HFE type 4) due to a new missense mutation in the FERROPORTIN 1 gene (SLC11A3) in a large French-Canadian family

We describe the identification of a new mutation (Y64N) in FERROPORTIN 1 gene (SLC11A3) detected in a large French-Canadian family with autosomal dominant iron overload and deposition mainly in Kupffer cells and macrophages. The Y64N mutation lies within the first transmembrane domain, whose role in iron transportation is further strengthened.

haematologica 2003; 88:824-826  
(<http://www.haematologica.org/824.htm>)

Hereditary hemochromatosis (HH) is usually transmitted as an autosomal recessive trait in which excessive iron absorption causes iron overload, primarily in parenchymal cells. It is mainly due to mutations HFE gene (hemochromatosis type 1),<sup>1</sup> but a minority of cases are associated with TFR2 gene mutations (hemochromatosis type 3).<sup>2</sup> In addition, a severe early onset form (hemochromatosis type 2) has been described and mapped.<sup>3</sup> More recently, an autosomal dominant form of HH has been described (HFE type 4) due to mutations in FERROPORTIN 1 (also known as solute carrier family 11, member A3, or SLC11A3).<sup>4-6</sup> We have identified a large French-Canadian pedigree affected by an autosomal dominant form of iron overload and characterized by 8 affected and 9 healthy members (Figure 1a). A summary of the most important iron parameters and clinical signs is reported in Table 1. The proband (2055) (now 28 years old) presented with fatigue, diarrhea and tremors. His serum ferritin concentration was 647 µg/L (normal range up to 400 µg/L) and transferrin saturation was 77% (normal range up to 45%). A magnetic resonance imaging (MRI) scan suggested iron accumulation in the liver and steatosis. A liver biopsy revealed iron deposition in both hepatocytes and Kupffer cells without fibrosis but with grade 4 at Perls' staining (Figure 1b). After one year of venesections (once

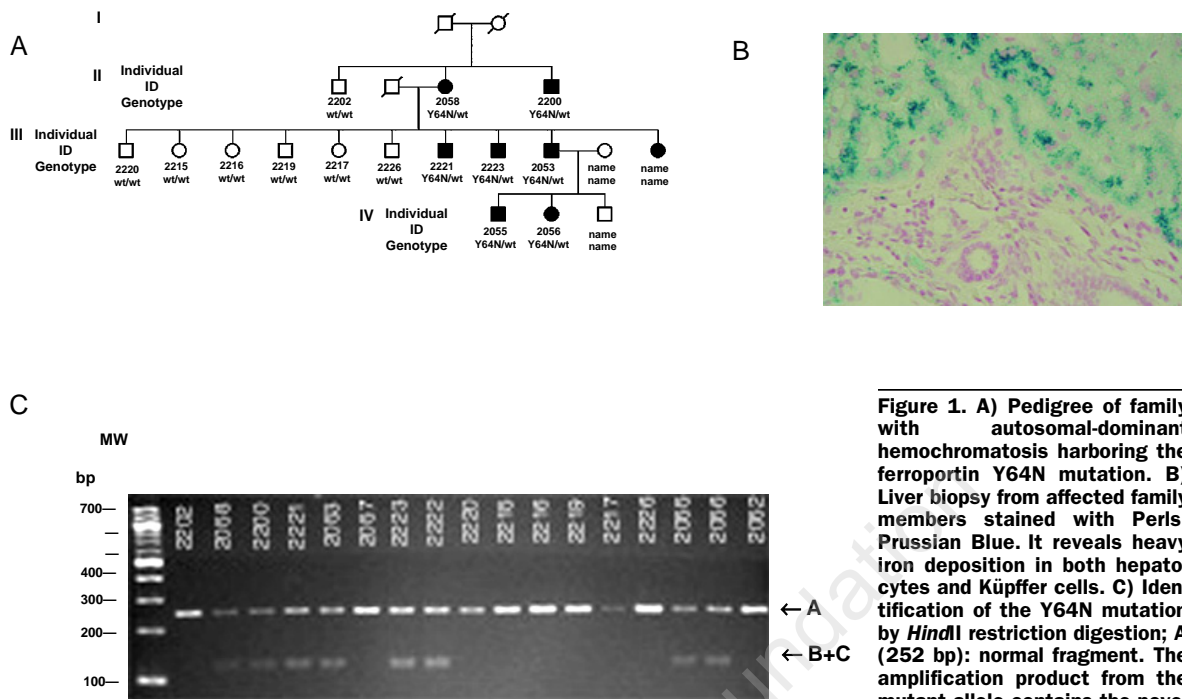
per week) (450 mL blood per week), the ferritin dropped to 44 µg/L with a transferrin saturation of 31%. The proband's father (2053) (now 59 years old) had a high serum ferritin concentration (1759 µg/L) and a transferrin saturation of 96%. Liver biopsy showed fibrosis and marked iron accumulation in hepatocytes and in macrophages and ferritin concentration fell to 15.5 µg/L after 2 years of phlebotomies. Elevated serum ferritin associated with increased transferrin saturation was found in 6 additional family members. All patients are now undergoing phlebotomies and none of them have any problem tolerating this therapy. Genomic DNA was obtained from all family members. Exclusion of HFE C282Y and H63D mutations was performed as previously described. Linkage to FERROPORTIN 1 locus (Lod score of 4.21 at recombination frequency of  $\tau=0$ ) has been demonstrated using the following markers: D2S2257, D2S350, D2S152, D2S118, D2S315 and D2S280. The following mutational screening of FERROPORTIN 1 gene, carried out by direct sequencing of its coding region and flanking intron-exon boundaries, led to the identification of a T to C change at nucleotide position 190 of the gene. This change corresponds to a substitution of an aromatic and hydrophobic tyrosine residue with a small and polar asparagine one at position 64 of the protein (Y64N). This mutation, which segregates with the disease and was not found in 100 normal controls, can be easily detected by RE digestion since it creates a new HincII restriction site (Figure 1c). According to consensus structural predictions the mutation is located in the first transmembrane domain of ferroportin 1 protein.<sup>8</sup>

Three other mutations in the human FERROPORTIN 1 gene have been so far reported.<sup>4,5,7</sup> Njajou et al. described a Dutch pedigree with a missense mutation (N144H) resulting in autosomal dominant hemochromatosis with significant iron overload treatable by phlebotomy. Montosi et al. reported a missense mutation (A77D) in an autosomal dominant Italian hemochromatosis pedigree, in which transferrin saturation was elevated in 8 of 15 family members. For N144H mutation a gain of function effect was proposed, while for A77D a loss of function and haploinsufficiency was suggested. Finally a common deletion, named V162del was described in cases with increased serum ferritin with either normal or raised transferrin saturation.<sup>6,8,9,10</sup> This mutation seems to lead to loss of function and deficiency in the release of iron from phagocytic cells, which becomes apparent on venesection. Serum ferritin concentra-

**Table 1. Clinical and biochemical features of 8 patients with a dominant form of hemochromatosis in a French-Canadian population.**

Patient no.	Sex	Age at diagnosis (years)	HFE genotype	Serum iron (µmol/liter)	Transferrin Saturation (fmol)	Serum ferritin (µg/L)	Hepatic abnormality	After phlebotomy serum (µg/L)
2053	M	56	wt/wt	44	0.96	1759	Fibrosis	16.5
2055	M	24	wt/wt	55	0.98	647	normal	43.6
2056	F	30	C282Y/wt	21	0.47	176	normal	Not yet deironed
2058	F	77	wt/wt	32	0.73	251	ND	192.4
2200	M	63	wt/wt	44	0.96	1197	ND	808.3
2221	M	52	wt/wt	39	0.91	2812	ND	Not yet deironed
2222	F	46	wt/wt	36	0.86	513	ND	Not yet deironed
2223	M	41	wt/wt	46	0.92	1867	ND	Not yet deironed

Normal values: serum iron, 9-29 µmol/L; Transferrin saturation, 0.20-0.45 fmol; serum ferritin 30-425 µg/L (men), 10-300 µg/L (women), ND: not determined.



**Figure 1. A) Pedigree of family with autosomal-dominant hemochromatosis harboring the ferroportin Y64N mutation. B) Liver biopsy from affected family members stained with Perls' Prussian Blue. It reveals heavy iron deposition in both hepatocytes and Kupffer cells. C) Identification of the Y64N mutation by *HindIII* restriction digestion; A (252 bp): normal fragment. The amplification product from the mutant allele contains the novel *HindIII* site resulting in cleavage into B (120 bp) and C (132 bp) fragments.**

tions reflect the increased level of storage iron in reticuloendothelial cells. In contrast, in HFE-related hemochromatosis, in which iron is initially confined to hepatic parenchymal cells, the transferrin saturation is elevated and the serum ferritin may be normal. All the mutations so far described are located at either on the transmembrane domains 1 and 3 or at the extracellular loop at the C-terminal end of helix 3. This finding suggests that this part of the ferroportin1 protein may act as functional binding sites for proteins, such as apotransferrin, ceruloplasmin, or hephaestin, which is important for the export of iron from the cell.<sup>11</sup> The present report further strengthens the importance of FERROPORTIN 1 in autosomal-dominant hemochromatosis and add new insights into the role of this gene in iron overload diseases.

Sylvain R. Rivard,\*° Carmela Lanzara,\*# Doria Grimard,\* Massimo Carella,° Hervey Simard,\* Romina Ficarella,\*# Raynald Simard,\* Adamo Pio D'Adamo,° Marc De Braekeleer,@ Paolo Gasparini\*#

\*Sagen Pharma, Canada; °Tigem, Telethon Institute of Genetics and Medicine; #Genetica Medica, Dipartimento di Patologia Generale, 2° Università degli Studi di Napoli; @Service de Cytogénétique, Cytologie et Biologie de la Reproduction, CHU Morvan et Université de Bretagne Occidentale, Brest, France

**Key words:** autosomal-dominant iron overload, FERROPORTIN 1, French-Canadian family.

**Correspondence:** Dr. Paolo Gasparini, MD, Tigem, Via Pietro Castellino n.111, 80131 Napoli, Italy. Phone: international + 39.081.6132227. E-mail: gasparini@tigem.it

### Manuscript processing

This manuscript was peer-reviewed by two external referees and by Professor Mario Cazzola, Editor-in-Chief. The final decision to accept this paper for publication was taken jointly by Professor Cazzola and the Editors. Manuscript received April 16, 2003; accepted May 27, 2003.

### References

1. Feder JN, Gnirke A, Thomas W, Tsuchihashi Z, Ruddy DA, Basava A, et al. A novel MHC class I-like gene is mutated in patients with hereditary haemochromatosis. *Nat Genet* 1996;13:399-408.
2. Camaschella C, Roetto A, Cali A, De Gobbi M, Garozzo G, Carella M, et al. The gene TFR2 is mutated in a new type of haemochromatosis mapping to 7q22. *Nat Genet* 2000;25:14-5.
3. Roetto A, Totaro A, Cazzola M, Cicilano M, Bosio S, D'Ascola G, et al. Juvenile haemochromatosis locus maps to chromosome 1q. *Am J Hum Genet* 1999;64:1388-93.
4. Njajou OT, Vaessen N, Joosse M, Berghuis B, van Dongen JW, Breuning MH, et al. A mutation in SLC11A3 is associated with autosomal dominant hemochromatosis. *Nat Genet* 2001;28:213-4.
5. Montosi G, Donovan A, Totaro A, Garuti C, Pignatti E, Casanelli S, et al. Autosomal-dominant hemochromatosis is associated with a mutation in the ferroportin (SLC11A3) gene. *J Clin Invest* 2001;108:619-23.
6. Devalia V, Carter K, Walker AP, Perkins SJ, Worwood M, May A, et al. Autosomal dominant reticuloendothelial iron overload associated with a 3 base pair deletion in the ferroportin1 gene (SLC11A3). *Blood* 2002;15:100:695-7.
7. Roetto A, Merryweather-Clarke AT, Daraio F, Livesey K, Pointon JJ, Barbabietola G, et al. A valine deletion of ferroportin 1: a

- common mutation in hemochromatosis type 4. *Blood* 2002;100:733-4.
- Camaschella C, Roetto A, De Gobbi M. Genetic haemochromatosis: genes and mutations associated with iron loading. *Best Pract Res Clin Haematol* 2002;15:261-76.
  - Cazzola M, Cremonesi L, Papaioannou M, Soriani N, Kioumi A, Charalambidou A, et al. Genetic hyperferritinaemia and reticuloendothelial iron overload associated with a three base pair deletion in the coding region of the ferroportin gene (SLC11A3). *Br J Haematol* 2002;119:539-46.
  - Wallace DF, Pedersen P, Dixon JL, Stephenson P, Searle JW, Powell LW, et al. Novel mutation in ferroportin 1 gene is associated with autosomal dominant hemochromatosis. *Blood* 2002;100:692-4.
  - Vulpe CD, Kuo YM, Murphy TL, Cowley L, Askwith C, Libina N, et al. Hephaestin, a ceruloplasmin homologue implicated in intestinal iron transport, is defective in the sla mouse. *Nat Genet* 1999;21:195-9.

### Induction of $\gamma$ -globin gene expression by tallimustine analogs in human erythroid cells

Modulation of  $\gamma$ -globin gene expression is of relevance for the treatment of  $\beta$ -thalassemia and sickle cell anemia. We studied the relationship between the structure of DNA-binding tallimustine analogs and their ability to induce erythroid differentiation of K562 cells. One of the most active analogs (compound 10710) was found to efficiently induce fetal hemoglobin (HbF) and  $\gamma$ -globin mRNA production also in normal human erythroid progenitors.

*haematologica* 2003; 88:826-827  
(<http://www.haematologica.org/826.htm>)

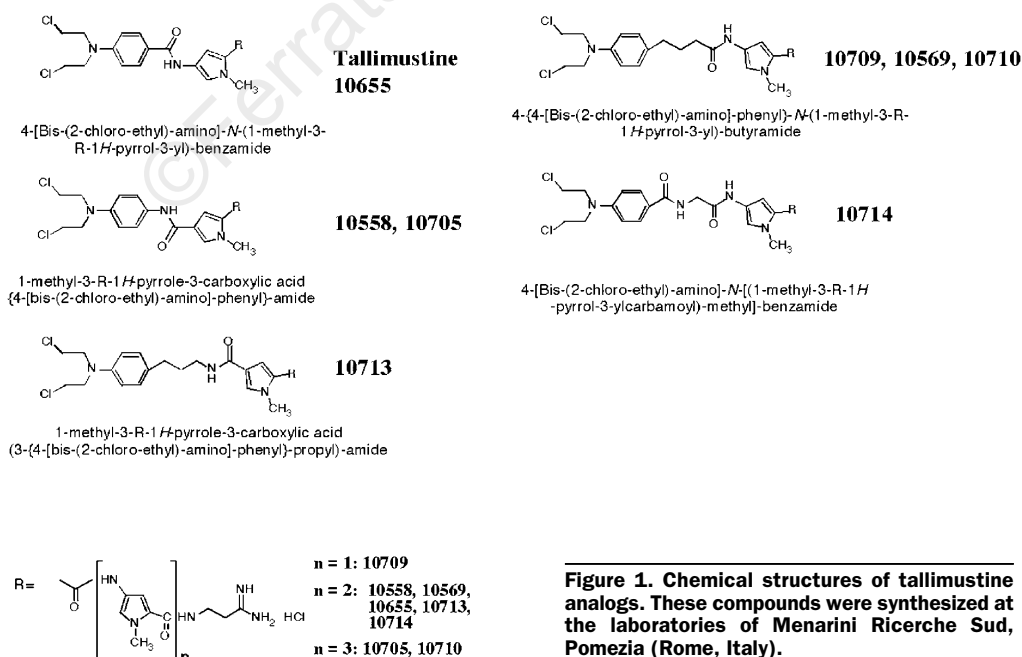
Drug-mediated modulation of interactions between DNA and nuclear proteins could represent a promising approach to control  $\gamma$ -globin gene expression and fetal hemoglobin (HbF) production.<sup>1-3</sup> This is of relevance for the treatment of  $\beta$ -thalassemia and sickle cell anemia,<sup>3</sup> since increasing HbF to 30% of the total hemoglobin production leads to an improvement of the clinical status.<sup>3</sup>

In the present study we first determined whether DNA-binding tallimustine analogs (Figure 1) induce erythroid differentiation of human erythroleukemia K562 cells. K562(S) cells were cultured, treated and analyzed as elsewhere reported.<sup>1,2</sup> The results (Figure 2A), obtained using compounds at concentrations causing 50% inhibition of cell growth, indicated that among the analogs carrying three pyrrole rings, tallimustine (10655) and 10569 exhibited high induction capacity. Addition of pyrrole rings significantly improved the inducing ability (comparing 10710 to 10569, 10569 to 10709 and 10705 to 10558 yielded p values < 0.03 by the one-way ANOVA test). The effect of the drugs on these cells was associated with production of Hb Portland and an increase in  $\gamma$ -globin mRNA content (data not shown).

The most active compound, 10710, was further studied in normal human erythroid precursors cultured in a two-phase liquid culture system.<sup>2,4,5</sup> Compounds were added on day 4-5 of phase II (when cells started to synthesize Hb). Hb was analyzed by high performance liquid chromatography (HPLC) on day 12 as elsewhere reported.<sup>4,5</sup> The representative example shown in Figure 2B demonstrates that the percentage of HbF with respect to total Hb (% HbF) increased from 1.8% (untreated cells) to 7.5% and 14.5% in cells treated with 0.5 and 0.75  $\mu$ M 10710, respectively. Lower levels of HbF were found with hydroxyurea, tallimustine, and 10569.<sup>2</sup>

Real-time quantitative reverse transcriptase polymerase chain reaction (RT-PCR) analysis of the globin mRNA content (Figure 2C)<sup>6</sup> indicated that 10710 increased both  $\gamma$ -globin and  $\beta$ -globin mRNA using GAPDH mRNA as a reference. However, the fold increase of  $\gamma$ -globin mRNA was significantly higher than that of  $\beta$ -globin mRNA (p < 0.07 by the one-way ANOVA test), suggesting a preferential induction of the expression of the  $\gamma$ -globin genes after treatment of human erythroid precursors with 10710. In contrast, the increase in  $\alpha$ -globin mRNA was only barely detectable (Figure 2C).

In conclusion, in this study we demonstrated that the number of pyrrole rings and the type of molecular bridge linking the alkylating moiety and the oligopyrrole carboxy-amido backbone affect the differentiation inducing ability of tallimustine analogs. The compound 10710 was the most efficient inducer of  $\gamma$ -globin mRNA synthesis and HbF production



**Figure 1. Chemical structures of tallimustine analogs. These compounds were synthesized at the laboratories of Menarini Ricerche Sud, Pomezia (Rome, Italy).**