

Autosomal dominant hereditary hemochromatosis associated with two novel Ferroportin 1 mutations in Spain

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Abstract

Hereditary hemochromatosis is a common disorder of iron metabolism most frequently associated with mutations in the HFE gene. Hereditary hemochromatosis may be caused by other genetic mutations including those in the *SLC40A1* gene. This report describes the clinical and laboratory findings of two Spanish families with autosomal dominant iron overload associated with previously unrecognized Ferroportin 1 mutations (p.R88T and p.I180T). The phenotype of iron overload in the patients carrying these mutations could correspond to the group of clinical mutations that lose their iron export function.

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Introduction

Hemochromatosis is the most common form of inherited iron overload in Caucasians. It is a group of disorders that result in impaired iron homeostasis leading to excessive intestinal iron absorption and accumulation in tissues. Untreated, the build-up of iron can lead to tissue damage including cirrhosis, diabetes mellitus, arthropathy, cardiomyopathy, endocrine abnormalities and hepatocellular carcinoma [1]. The vast majority of cases of clinical hereditary hemochromatosis are associated with homozygosity for the C282Y HFE mutation [2], although other HFE mutations may lead to iron overload [3]. This form of hemochromatosis (type 1) is characterized by a slow and progressive increase in plasma iron content, which may in adulthood lead to systemic iron loading of parenchymal cells, particularly hepatocytes and eventually to organ disease. Other forms include type 2 or juvenile hemochromatosis, which can be caused by mutations in the iron regulatory hormone hepcidin [4] or in the hemojuvelin gene [5]. Type 3 caused by mutations in

transferrin receptor 2 [6], and type 4 described as an autosomal dominant iron overload due to pathogenic mutations in the ferroportin gene (ferroportin disease) [7,8]. Ferroportin 1 (FPN1), also known as IREG1 or MTP1, is a molecule that plays an important role in iron export [9–11]. In almost simultaneous reports, Dutch [8] and Italian [7] groups described pedigrees with atypical hemochromatosis inherited as an autosomal dominant trait. The two groups found different missense mutations in the ferroportin (*SLC40A1*) gene. Mutations in the *SLC40A1* gene have since been reported independently by other investigators, and substantial clinical differences exist between the reported families [12–22].

We report the identification of two novel mutations (R88T and I180T) in the FPN1 gene, which are associated with autosomal dominant iron overload in Spain.

Materials and methods

Subjects

The proband of family A (II.1) was diagnosed with iron overload 12 years ago when he was 49 and since then he has

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undergone venesections. Serum iron, transferrin saturation and ferritin were measured in 12 members of his family. The proband of family B (II.2) was diagnosed with iron overload in a routine laboratory evaluation. Pedigrees of both families are shown in Fig. 1. All the families members studied gave informed consent in writing.

Iron studies

Transferrin saturation and serum ferritin were measured by standard methods in samples obtained from all members studied after an overnight fast. In those treated with phlebotomy, the total amount of iron removed was calculated as the number of phlebotomies (with 450 mL of blood drawn at each session) multiplied by 200 (the number of milligrams of iron removed per session). Liver biopsy samples were stained with hematoxylin and eosin and Perl's Prussian blue to evaluate the presence of iron. In selected family members, the hepatic iron concentration and the hepatic iron index were determined as previously described [23].

Clinical evaluation

The complete clinical records of all family members were revised, taking into account previous blood transfusion history, iron-containing medications, alcohol consumption, hepatitis B–C serology and the usual clinical complications of hemochromatosis.

Mutation analysis

The whole coding region, 5'UTR, 3'UTR and exon–intron boundaries of the FPN1 gene were analyzed. PCR amplifications were carried out as described [7,12]. Direct sequencing of PCR products was performed in both directions using dye-

terminator cycling sequencing on an automated sequencer according to the manufacturer (Applied Biosystems Abi Prism 310).

In family A, the c.263G > C substitution results in a loss of a *Bfa*I restriction site. In family B, the fragment containing the c.539T > C change was amplified using a mismatched exonic sense primer, 5'-GAA TGC CAC AAT ACG AAG AA-3', and an exonic antisense primer 5'-CAC AGC TAG AGC TGG GGT TT-3', which created a Tsp509I site in the normal allele, but not in the mutant allele. These changes in the restriction enzyme sites were used to perform the familiar studies and the screening of the control samples (DNA samples of 60 healthy blood donors).

HFE mutations were analyzed with the LightCycler equipment (Roche Diagnostics GmbH, Mannheim, Germany).

Results

Family A

The most relevant iron parameters of family A are shown in Table 1. The proband (II.1) was a 61-year-old man that came to the hospital because of an increase in his liver enzymes. The patient had a previous history of mild alcohol intake. His glucose metabolism was normal and there were no other clinical signs associated with hemochromatosis. Transferrin saturation (TS) was 91%, and serum ferritin 9075 µg/L. A liver biopsy revealed a severe iron deposit in both hepatocytes and Kupffer cells, with grade IV at Perl's staining. The pathologic study revealed fibrosis without cirrhosis. He had been treated by venesection since 1993. At the time of writing he has completed 66 procedures, and the ferritin now is 5200 µg/L. His wife and daughter showed normal iron parameters.

The proband has 5 first cousins (3 females and 2 males) from the paternal branch of the family. The three females had

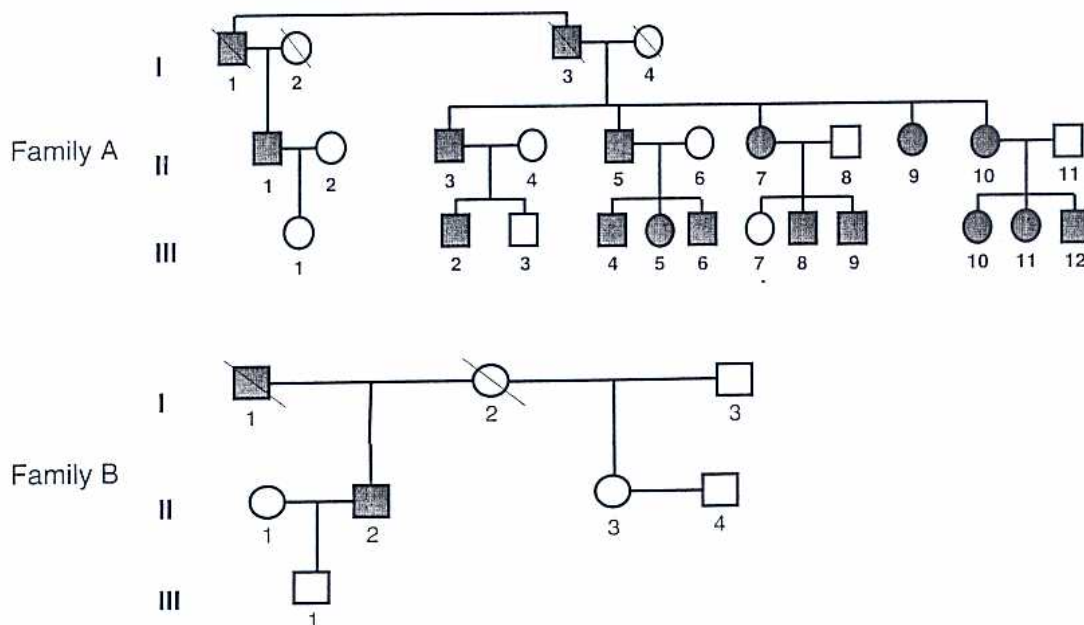


Fig. 1. Family pedigrees.

Table 1
Clinical, biochemical and genetic characteristics of family A patients

Patient	Age/Sex	Ferritin $\mu\text{g/L}$	TS %	HFE genotype	FPN1 genotype	Iron removed (gr)	Liver biopsy Histological grade of liver iron deposition
II-1	61/M	9075	91	wt/wt	Arg 88 Thr/wt	16.5	Grade IV mixed* iron deposit. Liver fibrosis. No cirrhosis
II-2	60/M	30	20	wt/h63d	wt/wt	NO	
II-3	56/M	2830	43	wt/wt	Arg 88 Thr/wt	9.5	Grade III mixed* iron deposit. Liver fibrosis. No cirrhosis
II-4	52/F	7	6	wt/wt	wt/wt	NO	
II-5	53/M	5291	70	wt/wt	Arg 88 Thr/wt	17.7	Grade III mixed* iron deposit. Liver fibrosis. No cirrhosis
II-6	47/F	16	26	wt/wt	wt/wt	NO	
III-1	20/F	25	12	wt/wt	wt/wt	NO	
III-2	22/M	1030	23	wt/wt	Arg 88 Thr/wt	NO	
III-3	17/M	20	17	wt/wt	wt/wt	NO	
III-4	23/M	1870	64	wt/wt	Arg 88 Thr/wt	11	Grade III mixed* iron deposit. No liver fibrosis.
III-5	18/F	724	60	wt/wt	Arg 88 Thr/wt	5	
III-6	15/M	386	14	wt/wt	Arg 88 Thr/wt	NO	

* Iron deposit in both, hepatocytes and Kupffer cells.

high levels of ferritin and were diagnosed with hemochromatosis. Two of the women were treated with phlebotomy. Five of their offspring had the same diagnosis and underwent phlebotomy. Unfortunately, these members of the family refused to participate in our study.

Patients II.3 and II.5, aged 56 and 53, respectively, were diagnosed with iron overload (Table 1). They did not have abnormal liver enzymes or hemochromatosis-associated complications. Patient II.5 had been diagnosed with viral hepatitis B and C, and his serology was positive. A liver biopsy was performed in both patients, which showed a pattern similar to that of the index case. The liver iron concentration was only measured in II.3, and was 200 $\mu\text{mol/g}$ dry weight, with a hepatic iron index of 4.2. Both underwent phlebotomies every two weeks (38 procedures in II.3 and 71 procedures in II.5) with current ferritin levels of 394 $\mu\text{g/L}$ in II.3 and 1041 $\mu\text{g/L}$ in II.5. Patient III.2 was a 22-year-old man who had a ferritin level of 1030 $\mu\text{g/L}$ with a normal TS, while his brother (III.3) showed an iron deficient pattern. Cases III.4, III.5 and III.6 had an iron overload status (Table 1). The liver biopsy performed in patient III.4 showed a pattern similar to those of his relatives, but with no fibrosis. Patients III.4 and III.5 currently undergo weekly phlebotomies (44 and 19 to date).

HFE genotyping (p.C282Y, p.H63D, and p.S65C) identified that an unaffected member of the family bears the p.H63D change in heterozygosis. (Table 1).

In family A, a novel single nucleotide substitution (c.263G > C) in exon 3 of the FPN1 gene was identified. The substitution results in a change of residue 88 from an Arginine to a Threonine (p.R88T). This mutation, which segregates with the disease and was not found in 60 healthy controls, can be easily detected by restriction enzyme digestion since it destroys a *Bfa*I restriction site.

Family B

In family B, the index case (II.2) was a 72-year-old man who was diagnosed with iron overload in a routine examination 4 years ago. He had no previous history of alcohol abuse or viral hepatitis. He suffered from diabetes mellitus type II

without other hemochromatosis associated complications. His TS was 42% and his serum ferritin concentration was 946 $\mu\text{g/L}$. The patient was put on a phlebotomy regimen, which was interrupted when he suffered a myocardial infarct. His father had died of hepatocellular carcinoma. His liver biopsy was revised and showed areas of hepatocarcinoma surrounded by cirrhotic liver tissue with huge parenchymal and reticuloendothelial iron deposits (grade IV). His wife and son had normal values of the iron metabolism parameters.

In this family, a novel single nucleotide substitution (c.539T > C) in exon 6 of the FPN1 gene was identified. The substitution results in a change of residue 180 from an Isoleucine to a Threonine (p.I180T). This mutation was not found in 60 healthy controls, can be easily detected by restriction enzyme digestion since it destroys a *Tsp*509I restriction site.

Discussion

To date, 17 different mutations in the FPN1 gene have been reported [11–13,15–22]. The vast majority (15/17) are missense mutations but one change in the 5'UTR region and a small deletion (p.V162del) have also been described. The reported mutations are usually exclusive to single families, although the p.V162del mutation recurs in different populations (UK, Australia, Italy and Greece).

Structural predictions of the FPN1 protein are still unclear. Some authors reported that the protein has 9–10 transmembrane domains [13] and although the reported mutations are scattered over the whole protein, the majority involves the regions between the first and the fifth transmembrane domains. According to these findings, this region may constitute a functional binding site for a regulatory protein crucial for the export of iron from the cell. Recently, Liu et al. [22] have determined the overall topology of the FPN1 protein and have found the existence of twelve transmembrane domains. These authors also indicate that all of the clinical mutations except C326S appear to be localized in regions of the protein exposed to cytosol and not to the external surface. Accordingly, it seems unlikely that hepcidin binding to FPN1 is disrupted by the mutations given that the hepcidin binding site is likely to be

extracellular. The two mutations reported in the present study probably cause structural perturbations: both, the p.R88T and the p.I180T replacements involve residues with different sizes and polarities. Until a consensus has been reached concerning the structure of FPN1, it is not easy to speculate on the relationship between location and functional alterations.

The clinical presentation observed in the type 4 hemochromatosis is heterogeneous: some patients present with macrophage iron deposition and high ferritin levels despite normal transferrin saturation, whereas others develop abnormalities similar to typical hemochromatosis (elevated TS and iron deposition in hepatocytes). Recent studies [24,25] postulate that ferroportin mutations fall into two categories. Those that traffic poorly to the cell surface and lose iron export function (associated with Kupffer cell iron deposition and normal transferrin saturation *in vivo*), and those that retain cell-surface expression and export function (associated with high transferrin saturation and iron deposition throughout the liver parenchyma). These authors find no evidence for multimerization of FPN1, and no evidence of physical or functional interaction between wild type and mutant forms of the protein. The recent report of De Domenico et al. [26] provides the molecular basis for the ferroportin-linked hemochromatosis. According to these authors, the disease is due to the FPN1 localization defect, resulting in a loss of iron export function or to resistance to negative regulation by hepcidin, resulting in a gain of iron export function. They demonstrate that FPN1 is multimeric and that the mutant FPN1 can affect the localization, the stability and the response to hepcidin of the wild-type protein. These authors point out that this dominant negative effect accounts for the dominant inheritance and for the different phenotypes characteristic of the ferroportin disease.

In family A carrying the p.R88T mutation, the affected patients present a TS that is not excessively high although it increases with age and iron loading. The three oldest patients suffer from a severe iron overload (very high ferritin levels at diagnosis, high number of phlebotomies with persistent elevated levels of ferritin). In these three patients, a pattern of iron loading in parenchymal and in reticuloendothelial cells with fibrosis but without cirrhosis is observed. In this family, the young patients have normal transferrin saturation levels and predominant Kupffer cell iron deposition, whereas those diagnosed at an older age have elevated transferrin saturation levels and the iron accumulation occurs in the hepatocytes. The pattern common to patients of family A is that despite presenting a very high iron overload, they only suffer from hepatic fibrosis, which does not develop into cirrhosis. In the case of the p.I180T mutation, the clinical expression is similar to that of the aforementioned p.R88T mutation albeit with a milder clinical presentation. Despite the lack of functional studies of these mutations, their phenotypic expression could correspond to the group of clinical mutations that lose their iron export function.

Acknowledgments

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