Primary Iron Overload With Inappropriate Hepcidin Expression in V162del Ferroportin Disease

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Ferroportin disease (hemochromatosis type 4) is a recently recognized disorder of human iron metabolism, characterized by iron deposition in macrophages, including Kupffer cells. Mutations in the gene encoding ferroportin 1, a cellular iron exporter, are responsible for this iron storage disease, inherited as an autosomal dominant trait. We present clinical, histopathological, and radiological findings in a family with the most common ferroportin mutation, V162del. In the index case, the disorder is characterized by abundant deposition of hemosiderin in all tissues investigated (mesenteric lymph node, liver, gastric and duodenal mucosa, and also in squamous cell carcinoma of the lung). The radiological findings indicated the presence of excess iron in bone marrow and spleen. Despite a significant burden of iron, no features of chronic liver disease were found in affected members of the family, including individuals aged up to 80 years. Hyperferritinemia greater than 1,000 μg/L was a penetrant biochemical finding before the second decade in life and was associated with significantly increased serum concentrations of pro-hepcidin that correlated positively with urinary hepcidin concentrations. In conclusion, the systemic iron burden in ferroportin disease is not a sufficient cause for chronic liver disease. In patients with most, but not all, ferroportin mutations, retention of iron in macrophages of the liver and other organs may protect against damage to parenchymal cells. Finally, macrophage iron storage in ferroportin disease is associated with elevated serum pro-hepcidin levels. (HEPATOLOGY **2005;42:466-472.**)

Mutations in the human gene encoding ferroportin 1 (*FPN1/IREG-1/MTP-1/SLC40A1*) are associated with an unusual iron overload syndrome, recently named hemochromatosis type 4 or ferroportin disease.[1-9] Clinically, ferroportin disease is rarely associated with hepatic cirrhosis, and the principal pathological finding is iron deposition predominantly in cells of monocytic macrophage lineage—mainly and conspicuously Kupffer cells within the liver.

The most common *FPN1* mutation is deletion of a valine at position 162 and has been identified in patients of various ethnic backgrounds.[3,8,10,11] Deletion of this amino acid causes loss of function and when studied *in vitro* causes accumulation of iron in cultured cells.[12] FPN1 is a putative transmembrane iron channel implicated in the egress of iron from duodenal enterocytes, macrophages, hepatocytes, and placenta (reviewed in McKie and Barlow[13]). Impaired iron export from macrophages in patients with mutations in the *FPN1* gene has been proposed as the explanation for the accumulation of iron that occurs in organs containing abundant macrophages such as the liver, spleen, and bone marrow.[14]

The differential diagnosis of iron accumulation in macrophages also includes the anemia of inflammation, in which anemia is associated with inappropriate retention of iron in macrophages and hepatocytes despite iron-restricted erythropoiesis.[15]

Hepcidin, which is expressed in the liver, heart,[16] and kidney,[17] is the key mediator of anemia of inflammation,[18,19] and synthetic hepcidin was shown to interact physically with ferroportin in a cellular overexpression

Abbreviations: MRI, magnetic resonance imaging; CT, computed tomography; PCR, polymerase chain reaction.

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system, causing internalization and degradation, and decreased export of iron.[20] Because hepcidin expression increases in response to iron loading (reviewed in Ganz[21]), a feedback loop in which body iron homeostasis is controlled by hepcidin has been postulated.

The important physiological function of hepcidin is further demonstrated by the observation that homozygous mutations in hepcidin have been identified in 4 pedigrees with juvenile hemochromatosis.[22-25] Moreover, targeted disruption of the hepcidin gene in mice induces severe iron overload of the liver and pancreas, resembling that observed in human hemochromatosis.[26] In the classical HFE1-associated hemochromatosis (hemochromatosis type 1), as well as in rare hemochromatosis due to homozygous mutations in genes encoding transferrin receptor 2 (hemochromatosis type 3) or hemojuvelin (hemochromatosis type 2), liver hepcidin mRNA expression or urinary hepcidin excretion appears to be inappropriately low, a finding that may explain the persistently increased absorption of iron in the face of systemic iron excess in these diseases.[27-29] We present the clinical, radiological, histological, and biochemical findings in a pedigree affected by ferroportin disease (hemochromatosis type 4). Extrahepatic iron deposition in an adult man led to the diagnosis and, as a result, investigations identified 4 more affected individuals. In all affected individuals, serum pro-hepcidin concentrations before iron depletion therapy were found to be increased more than threefold above the mean concentration in healthy persons, and a statistically significant correlation between serum pro-hepcidin concentrations and urinary hepcidin excretion was found.

Patients and Methods

Informed consent was obtained from all patients involved in the study. DNA for genetic testing was prepared from venous blood samples using the QiaAMP DNA mini Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. Genetic testing for C282Y mutation in the HFE gene was performed as previously described.[30] Molecular analysis of the human ferroportin gene (*FPN1, HFE4*) was carried out as follows: each exon, including intron–exon boundaries, was polymerase chain reaction (PCR) amplified, and direct sequencing analysis carried out on an ABI Prism 310 automated sequencer. Primers and PCR conditions were used as described in Cazzola et al.[8] Serum iron parameters were determined using standard laboratory techniques (Abbott, Vienna, Austria).

Pro-hepcidin ELISA was purchased from DRG Diagnostics (Marburg, Germany) and carried out following the maufacturer's instructions and as previously described.[31] For quantification of serum pro-hepcidin, frozen serum samples were used, avoiding repeated freeze–thaw cycles, which can cause significant pro-hepcidin degradation. Tissues were embedded in paraffin and stained with hematoxylin-eosin according to standard techniques. Perls' Prussian blue stain was carried out as described by Perls[32] and Stevens.[33]

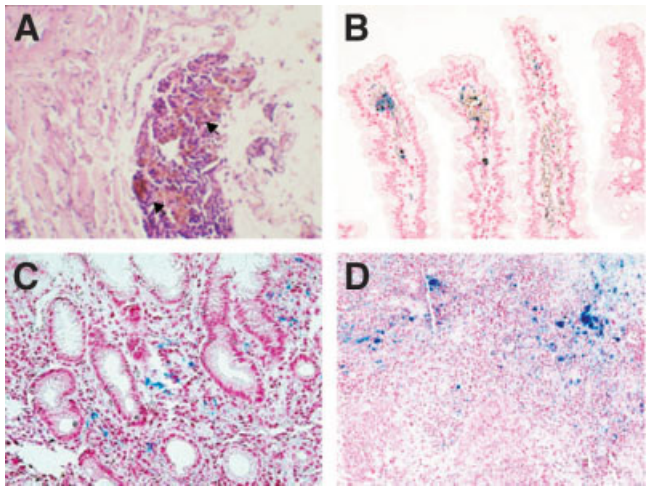

Fig. 1. (A) Mesenteric lymph node with brown hemosiderin deposits on HE staining (arrows) (original magnification ×200). (B) Duodenal biopsy–stainable iron in villous macrophages (Perls' Prussian blue stain; original magnification ×100). (C) Gastric antrum biopsy–stainable iron in gastric mucosa (original magnification ×200). (D) Squamous cell carcinoma of the lung–stainable iron in tissue macrophages (Perls' Prussian blue stain; original magnification ×100).

Measurement of urinary hepcidin concentration was carried out as described elsewhere.[34]

Results

Index Case. Patient no. 1 underwent cholecystectomy for symptomatic cholelithiasis in 1986, when histological examination of a macroscopically suspicious mesenteric lymph node indicated marked iron deposition (Fig. 1). The 68-year-old patient was subsequently found to have a serum ferritin of 5,265 μg/L (reference range, 5-307 μg/L) and a serum transferrin saturation of 37.5% (reference range, 20%-50%). Initial genetic investigations for hereditary hemochromatosis type 1 showed the HLA phenotype A2/A3, B7/B40, BW 6/CW 3, of which A3 and B7 are known to be associated with this hemochromatosis type 1 (HFE1). However, *HFE1* genotyping, which was performed in 1997, showed no pathological mutations in the *HFE1* gene. The patient refused liver biopsy, but magnetic resonance imaging showed abnormal signals in the liver, spleen, and bone marrow, indicating tissue iron storage.

In light of these findings, secondary causes for iron overload, such as heavy alcohol consumption or iron-

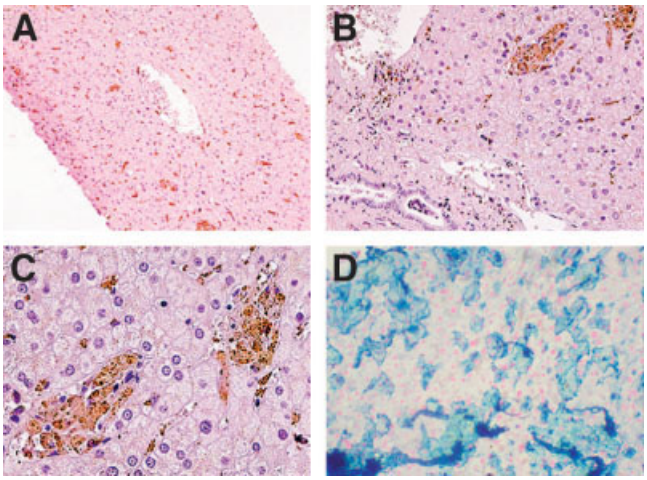

Fig. 2. (A-C) Liver histopathology of patient no. 5. There were hemosiderin deposits in the liver sinusoids, but no hemosiderin was found in hepatocytes. (D) Perls' Prussian blue stain of the liver showing considerably less stainable iron in hepatocytes than in Kupffer cells. Original magnification ×50 (A), ×200 (B), ×400 (C), ×500 (D).

loading anemias, were excluded. Neither was there any evidence of chronic inflammation, which could cause sequestration of iron in macrophages. Therapeutic phlebotomy was started after serum iron parameters remained unchanged over 5 years. Phlebotomy was well tolerated, and a total of 22 L of blood were removed over a period of 22 months (equivalent to approximately 12 g iron). During this treatment, serum ferritin decreased from 5,265 μg/L to 1,140 μg/L. Although hemoglobin levels were never below 12 g/dL, therapeutic phlebotomy was stopped for 8 months because of non-compliance, which resulted in an increase in serum ferritin concentration to 2,111 μg/L. On follow-up hemoglobin, aminotransferases, bilirubin, and coagulation tests remained normal; furthermore, sonography and esophagoscopy showed neither signs of chronic liver disease nor portal hypertension, respectively.

At the age of 77 years, the patient complained of gradually worsening shortness of breath: a diagnosis of squamous carcinoma of the bronchus was made by biopsy of a tumor obstructing a main bronchus. At the age of 80 years, the patient died of complications of the carcinoma after a course of palliative radiation therapy.

On family screening for iron overload, 2 sons of the index case were found to have iron overload (Fig. 2), one aged 29 (patient no. 5) with a serum ferritin of 4,935 μg/L and one at the age of 50 (patient no. 4) with a serum ferritin of 7,239 μg/L. The latter finding may have been exacerbated by heavy consumption of alcohol before presentation. Of the 2 remaining sons, 1 had a normal serum ferritin and the other was not available for a medical investigation. In the third generation of this family, the 17-year-old twin sons of patient no. 3 were found to have serum ferritin concentrations of 1,150 μg/L and 1,324 μg/L. The serum iron parameters of all family members available for investigation are displayed in Table 1.

In all affected family members, phlebotomy was well tolerated, and in patient no. 5 fortnightly therapeutic phlebotomy for 23 months resulted in a decline of serum ferritin concentrations from 4,935 μg/L to 345 μg/L. Complete hematological and biochemical parameters remained within the normal range during follow-up. In all other cases, the diagnosis was established only recently, and hence data on the effects of prolonged phlebotomy are not yet available.

Histopathology. Histological examination of the macroscopically suspicious mesenteric lymph node, which was removed during the cholecystectomy, showed hemosiderin deposits (Fig. 1A).

The patient refused liver biopsy, which was offered after identification of stainable iron in the lymph node that led to the detection of hyperferritinemia. However, during follow-up, gastroduodenoscopy was performed at the age of 65 years for upper abdominal pain. Histological examination of the duodenal biopsy showed stainable iron in the villous macrophages. Furthermore, histological examination of the squamous cell bronchial carcinoma

Table 1. Serum Iron Parameters of All Family Members Available for Detailed Investigation

Individual No.	Generation	Iron (μmol/L)	Ferritin (μg/L)	Transferrin (g/L)	Transferrin Saturation (%)	Hb (g/dL)	Ferroportin 1 Genotype	Iron Removed	HFE Genotype	Age (yrs)
1	*I*	*14.2*	*5,265*	*2.13*	*37*	15.0	*V162del*	*~12g*	*wt/wt*	*68*
2	I	20.1	152	1.91	28	13.4	wild type		wt/wt	78
3	I	19.2	253	2.21	36	14.9	wild type		wt/wt	66
4	*II*	*19.2*	*6,563*	*2.2*	*35*	15.4	*V162del*	*~15g*	*wt/wt*	*51*
5	*II*	*10.2*	*4,935*	*2.49*	*23*	15.5	*V162del*		*wt/wt*	*26*
6	II	23.2	280	2.34	31	15.2	wild type		wt/wt	24
7	*III*	*24.1*	*1,150*	*2.58*	*37*	14.9	*V162del*		*C282Y/wt*	*17*
8	*III*	*13.9*	*1,314*	*2.3*	*24*	13.8	*V162del*		*C282Y/wt*	*17*
9	III	27.9	18	2.9	18	14.1	wild type		*C282Y/wt*	19

NOTE. Affected individuals are listed in italics.

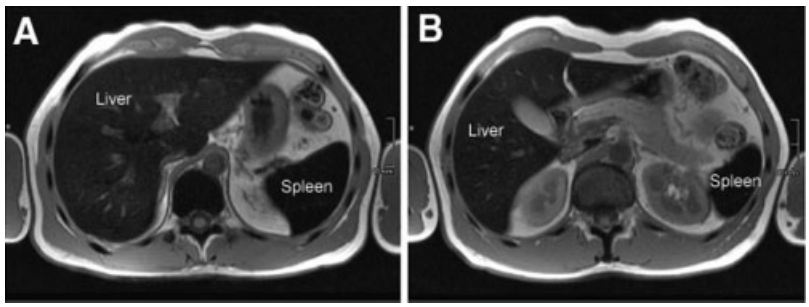

Fig. 3. Images of a T2-weighted MRI scan of the abdomen of patient no. 5. Note the low signal intensity in liver, spleen, and bone marrow with normal signal intensities in pancreas and kidneys. (A) Abdomen transversal section showing iron accumulation in the liver and spleen. (B) Abdomen transversal section showing no iron accumulation in pancreas and kidneys.

(epidermoid cellular) also reflected increased stainable iron in tumor-associated macrophages (Fig. 1D).

The liver biopsy of patient no. 2 at the age of 29 years is shown in Fig. 2, where excessive brown deposits (hemosiderin) within the liver were found. Unlike patients with classical *HFE1*-associated hemochromatosis, no hemosiderin and little stainable iron were detected in hepatocytes; hemosiderin was mainly found in the sinusoids. Perls' Prussian blue stain of the liver confirmed severe iron accumulation, predominantly in Kupffer cells (Fig. 2B,D). No signs of inflammatory liver disease or fibrosis were found. Taken together, these findings demonstrate that ferroportin disease (hemochromatosis type 4) caused by the V162del mutation is a systemic disorder in which the macrophage is the principal cell affected.

Radiology. To exclude hepatocellular carcinoma at the time of presentation with severe iron overload and to further assess the severity of hepatic iron overload, magnetic resonance imaging (MRI) was performed. The signal intensity of T2-weighted MRI scans of the liver was significantly reduced in patient 1 and patient 5, which accords with the histological findings that demonstrated heavy iron deposits in the liver. Furthermore, T2 signal intensities in spleen and bone marrow were also reduced in both patients, suggestive of iron deposition in these macrophage-rich organs (Fig. 3). Computed tomography scanning of the abdomen was carried out during surveillance for hepatocellular carcinoma every 3 years, on which radiological changes, compatible with iron deposition in liver, spleen, and lymph nodes, were found (not shown). Signal hyperintensity of the liver with negative contrast of the liver veins and signal hyperintensity of the spleen are known to be nonspecific CT findings, which can be due to iron deposition.

Genetics. The pedigree is displayed in Fig. 4A. The inheritance pattern is characteristic of an autosomal dominant trait, with affected individuals represented in all 3 generations. Sequencing of the ferroportin gene revealed the most common ferroportin mutation (a deletion of 3 base pairs in exon 5), which results in loss of a valine in position 162 (V162del) of the predicted ferroportin1 protein. The gene mutation is fully penetrant at the biochemical level, with hyperferritinemia present in all heterozygous family members. Unaffected family members in all 3 generations were found to have normal serum ferritin concentrations. Mutations in all other exons of the ferroportin gene were excluded by complete sequencing of the coding region. In summary, the mutation was fully penetrant and co-segregated with the phenotype of hyperferritinemia and normal serum transferrin saturation in this family.

Serum Pro-hepcidin. In the 3 affected individuals from whom pre-treatment serum samples were available serum pro-hepcidin concentrations were 288 ng/mL, 429 ng/mL, and, 649 ng/mL, all of which are above the reported range in healthy individuals (52-153 ng/mL).[31] Furthermore, these values are even higher than the reported median for chronic renal insufficiency (148 ng/mL).[31] In the 2 affected family members (patient 1 and patient 2) from whom serum samples were available only after initiation of treatment, serum pro-hepcidin concentrations were determined at 68 ng/mL and 159 ng/mL, respectively. During venesection therapy, the concentration of serum pro-hepcidin gradually decreased.

As shown in Fig. 5B, a statisticaly significant correlation between serum pro-hepcidin concentration and urinary hepcidin concentration was found (Deming regression analysis correlation coefficient 0.98, $P < .001$).

Discussion

Hyperferritinemia is a common finding in clinical practice, and the differential diagnoses include many clinical conditions such as chronic liver disease, iron-loading anemias, inflammation, and malignant diseases, in all of which serum iron and transferrin saturation are usually decreased. *HFE1*-associated hemochromatosis is a frequent cause of hyperferritinemia but usually is associated with elevated serum iron concentrations and transferrin saturation. Recent advances in the study of hereditary iron overload syndromes have shown that hereditary hemochromatosis can be caused by homozygous mutations in several other genes (*TfR2, HJV, HAMP*)[35] and

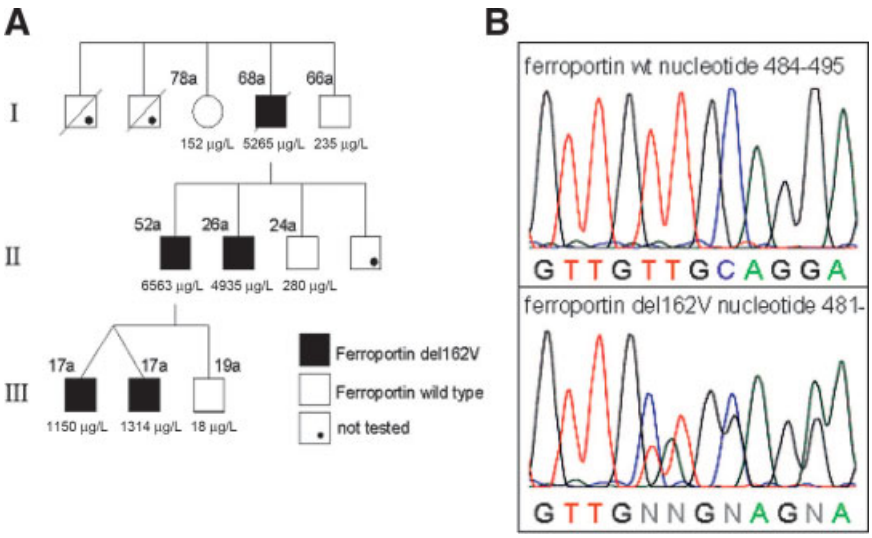

Fig. 4. (A) Pedigree of a family with iron storage associated with a mutation in the human ferroportin gene. Affected individuals, heterozygous for the V162del mutation, are represented by solid symbols, which are labeled with age at diagnosis (above) and serum ferritin values (below). (B) DNA Sequence electropherograms of an unaffected (above) and an affected individual (below). Note that GTT (484-486) is missing, resulting in an in-frame deletion on one allele and loss of a valine residue at position 162 of the mature protein.

may be associated with more complex compound genotypes.[35,36]

In this pedigree, the abnormalities were associated with the most common ferroportin mutation, V162del. On histological examination of various tissues, iron deposition was found not only in Kupffer cells but also in many other types of macrophages. Stainable iron was detected in duodenal villous macrophages, lymph node macrophages, and the tumor macrophages associated with squamous cell carcinoma of the lung. Systemic macrophage iron overload was further supported by the findings of the CT and MRI scans, where the radiological abnormalities were strongly indicative of increased tissue iron in spleen and bone marrow.

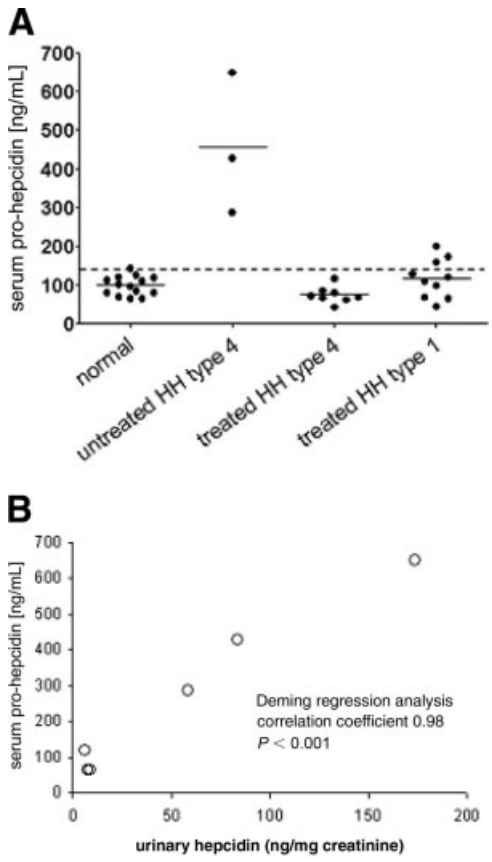

Fig. 5. (A) Serum pro-hepcidin concentrations in healthy individuals, untreated ferroportin disease (hemochromatosis type 4 [HH type 4]), treated ferroprotin disease, and treated adult hemochromatosis type 1. Broken line indicates the highest concentration reported in healthy individuals' normal (153 ng/mL).[30] (B) Correlation between serum pro-hepcidin and urinary hepcidin concentrations.

The total burden of iron in the body was apparently very high in at least 2 affected members of this pedigree, as suggested by high serum ferritin concentrations and by estimation of mobilizable iron during therapeutic phlebotomy. The high iron burden is notable, because no clinical, histological, biochemical, or radiological signs of liver disease or extrahepatic manifestations of hemochromatosis could be found; cardiomyopathy, diabetes mellitus, arthropathy, hypogonadism, or endocrinopathy (reported clinical features in patients with the N144H mutation in the ferroportin gene[9]), were significant by their absence. Furthermore, unlike other reported cases of ferroportin disease (hemochromatosis type 4)[9,10] in patients with the V162del mutation, therapeutic phlebotomy was well tolerated and iron could be readily mobilized without induction of anemia.

The absence of any clinical manifestation of iron-storage disease in this pedigree even in individuals of advanced age, however, raises the question as to whether phlebotomy should have been started at all. Liver fibrosis and cirrhosis are rare in hemochromatosis type 4 and have been mostly associated with a different class of ferroportin mutations, which result in a phenotype similar to classical hemochromatosis, with iron deposition in hepatocytes and increased transferrin saturation.[3-10,37,38] The severity

of iron deposition in Kupffer cells and the absence of stainable iron in hepatocytes in the liver biopsy for patient no. 5 suggests that iron loading of macrophages is well tolerated, but equivalent loading of hepatocytes may be the cause of fibrosis and cirrhosis in classical hemochromatosis.[39]

Relative hepcidin deficiency has been reported in *HFE1*-associated hemochromatosis, juvenile hemochromatosis, and TfR2-associated hemochromatosis.[29,31,34] Here we provide a report of serum pro-hepcidin expression in ferroportin syndrome (hemochromatosis type 4) in which we demonstrate a strong correlation between serum pro-hepcidin concentration and urinary hepcidin concentrations in individuals with elevated hepcidin expression. In contrast to other types of hemochromatosis, serum pro-hepcidin concentrations in patients heterozygous for the V162del mutations in the ferroportin gene were found to be markedly increased, which is in accordance with a recent study demonstrating increased urinary hepcidin concentrations[34] in other patients with this mutation. The increase, however, appears to be dynamic so that hepcidin remains responsive to changes in iron status, because phlebotomy resulted in a gradual decrease in serum pro-hepcidin in at least 2 patients. The processing of pro-hepcidin to hepcidin, however, has not yet been characterized, and it is uncertain as to whether serum pro-hepcidin and urinary hepcidin also correlate in other disorders of iron homeostasis.

One explanation for the coexistence of systemic iron overload with high hepcidin concentrations, which should reduce iron absorption, is that the hepcidin signal can be overridden by another signal that induces iron absorption. In our youngest patients, aged 17 years, serum ferritin concentrations were greater than 1,000 μg/L, and serum pro-hepcidin concentrations also were increased. Because we observed an age-dependent increase in serum ferritin and pro-hepcidin, iron absorption in these patients was still inappropriately high, even though serum (pro-)hepcidin concentrations were elevated. In the light of this observation, we question whether there is indeed an unidentified "erythroid regulator" inducing iron absorption, as proposed by the late Clement Finch.[40] If so , such an entity appears to override the effect of high (pro)-hepcidin concentrations.[40] However, the severe iron overload phenotype in patients with hepcidin deficiency[22,23] and the severe iron deficiency that develops in mice[41] or humans[42] overexpressing hepcidin strongly suggests that this peptide is the principal determinant of iron absorption in mammals.

Recently, Nemeth et al.[20] found that synthetic hepcidins bind to ferroportin expressed as a fusion protein in cultured cells, thereby inducing ferroportin internalization and its subsequent degradation.[20] As shown *in vitro,* if the V162del ferroportin variant is overexpressed in cell lines, no such internalization can be induced by synthetic hepcidin.[43] However, because hemochromatosis type 4 has only been recognized in individuals heterozygous for mutations in the human ferroportin gene, the contribution of ferroportin expressed from the coexistent wild-type allele to the disease phenotype cannot readily be unraveled.

There are indications that type 4 hemochromatosis itself is a heterogeneous disorder whose phenotype depends on the nature of the mutation affecting the ferroportin molecule. Thus, at least some patients with mutations in amino acid 144 have been reported to show increased transferrin saturation and evidence of liver disease, without any other known causes for systemic iron overload or hepatocyte iron loading. Ferroportin is highly expressed in duodenal enterocytes, hepatocytes, and macrophages (as well as in the placenta). It is conceivable that certain mutations do not induce a major bottleneck to iron release from macrophages but still display resistance to the inhibitory effects of hepcidin on iron export. In such patients, the phenotype could be dominated by excessive intestinal iron absorption unresponsive to increased hepcidin concentrations. Other patients, including those harboring V162del mutations, may be primarily affected by the consequences of functional deficiency of ferroportin in macrophages. Paradoxically, retention of iron in macrophages may protect these patients against injury to parenchymal cells in the liver and other organs, even when intestinal absorption and systemic iron load are also increased.

In summary, we report here detailed studies in a pedigree with an unusual presentation of hemochromatosis type 4 that highlight the systemic nature of the disorder. Despite a greatly increased burden of iron, chronic liver disease is not the clinical hallmark of the disorder. Finally, we report high serum pro-hepcidin concentrations that were apparent in the early stages of hemochromatosis type 4—an unexpected finding, because aquisition of iron by absorption at this stage of the disorder is increased.

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