Iron overload due to mutations in ferroportin

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Iron overload disease due to mutations in ferroportin has a dominant inheritance and a variable clinical phenotype, such that some patients show early Küpffer cell iron loading and low transferrin saturation, while others show hepatocyte iron loading and high transferrin saturation. Studies expressing ferroportin mutant proteins in cultured cells have shown that mutant proteins fall into two main classes; proteins that do not localize to the cell surface and are unable to export iron, and those that localize to the cell surface but are unable to respond to the antimicrobial peptide hepcidin. Patients with mutant ferroportin proteins that do not localize to the cell surface show typical ferroportin disease with low transferrin saturation and early Küpffer cell iron loading, while patients with mutant proteins unable to respond to hepcidin show high transferrin saturation and early hepatocyte iron loading similar to *classic* hereditary hemochromatosis. The dominant genetic transmission of ferroportin-linked disorders is explained by the *in vitro* data, which suggest that ferroportin is a multimer and that the behavior of the mutant protein can affect the behavior of the wild type protein.

Key words: hemochromatosis, iron, ferroportin, mutations, disease, hepcidin, subcellular localization, human.

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Mammalian iron homeostasis is dominated by the fact that there is no normal excretory route for iron. Iron homeostasis is regulated at the level of intestinal absorption by the body's demand for iron. In normal adults, iron absorption matches the rate of iron loss due to desquamification or intestinal sloughing. Iron absorption can be increased in response to increased need deriving from events such blood loss. Under most conditions, however, the rate of iron absorption by the intestine is low (1-2 mg/day) relative to total body iron (4.0 grams). Most of the iron that enters plasma results from iron export by macrophages as they recycle iron following the ingestion of aged or damaged red blood cells. Hereditary hemochromatosis (HH) presents as a disease of excessive parenchymal iron accumulation resulting from genetic disorders of iron absorption. As little as a decade ago, HH was relatively undifferentiated and was used as a generic label for primary iron overload diseases. During the past decade enormous progress has been made in understanding the genetic basis of iron overload diseases and the molecular events that result in tissue and cellular iron overload.

Absorption of dietary iron by the intestine, as well as iron recycling by the macrophage, is regulated by several different physiological cues including iron load, erythropoiesis, hypoxia and inflammation. An essential component in the regulation of iron homeostasis is the secretion of the liver antimicrobial peptide hepcidin.[1,2] Hepcidin is a 25 amino acid peptide secreted by the liver into the circulation. Hepcidin production is regulated transcriptionally, although the specific transcription factors remain unknown. Importantly, hepcidin synthesis is inversely correlated with iron demand; transcription of hepcidin is increased when iron stores are high and, conversely, decreased when iron stores are depleted. Hypoxia or increased erythropoiesis leads to decreased hepcidin production. Conditions of chronic inflammation, such as arthritis or cancer-associated inflammation, lead to increased production of hepcidin and decreased iron absorption resulting in an iron-limited erythropoiesis, referred to as the anemia of chronic inflammation.

Much of the regulation of iron homeostasis can be explained by the observation that the molecular target of hepcidin is an iron transporter present on the surface of all iron-exporting tissues. Ferroportin-1 (Fpn, also known as SLC40A1, IREG1, MTP1) is the only identified transporter that exports iron from cells to plasma.[3-5] Fpn is a membrane protein with 12 predicted transmem-

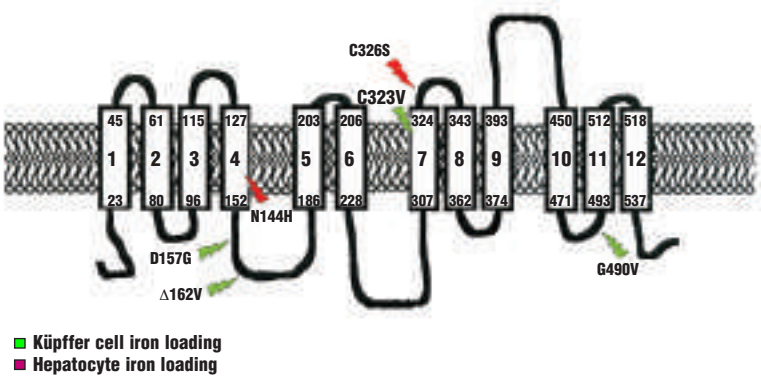

Figure 1. Predicted structure of ferroportin. Fpn is a membrane protein with twelve predicted transmembrane domains. Several mutations in the Fpn gene have been reported. Mutations that lead to Kupffer cell iron loading are shown in green and those that lead to hepatocyte iron loading are shown in red.

brane domains. Figure 1 is a schematic of a possible structure, although the structure requires further confirmation.[6] Fpn is found on the surface of macrophages, Küpffer cells, hepatocytes, intestinal enterocytes and placental cells (Figure 2). Fpn expression is regulated at several different levels. Fpn shows transcriptional regulation, as it is highly expressed in *professional* iron exporters such as duodenum, macrophages and placenta. Fpn contains an iron responsive element (IRE) in the 5' untranslated region of the mRNA, which subjects it to regulation by the iron responsive protein (IRP).[7] Thus, under conditions of cellular iron loading there should be increased expression of Fpn, leading to iron export. Most importantly, Fpn is regulated post-translationally by hepcidin. Hepcidin binds to Fpn leading to its internalization from the cell surface and its degradation in lysosomes.[8] Under conditions of high liver iron stores, production of hepcidin will lead to the down-regulation of Fpn on iron exporting tissues (e.g. intestine and macrophages), limiting entry of iron into plasma. Conversely, decreased expression of hepcidin results in increased iron export to plasma.

Primary iron overload disorders can be explained in terms of decreased hepcidin permitting increased iron export. Increased plasma iron first binds to transferrin resulting in increased transferrin saturation. Plasma iron in excess of transferrin is then accumulated by transferrin-independent iron transport system(s), which are prevalent in parenchymal tissue cells such as hepatocytes, cardiomyocytes, adrenal gland cells and the β-cells of the pancreas. It is excessive iron absorption in these cell types that is responsible for the clinical symptoms of *traditional* HH. HH is classified into four groups based upon the underlying genetic mutation.[9-11] The most clinically severe form of HH, juvenile hemochromatosis or HH type 2, results from mutations in the hepcidin or hemojuvelin genes. Other mutations that lead to HH, mutations in the HFE gene (HH type 1) or the transferrin receptor-2 gene (HH type 3) all affect hepcidin production. The amount of hepcidin produced in individuals with these three forms of HH is inadequate relative to iron stores, resulting in increased iron absorption. While the severity of iron overload in HH types 1-3 varies, the mode of genetic transmission and pathological findings are similar. The three disorders are transmitted as genetic recessive conditions and most iron is deposited in the liver and other parenchymal tissues.

There is one exception to the paradigm that decreased hepcidin production results in an iron overload disorder: a genetic form of iron overload disease resulting from mutations in Fpn (HH type 4).[12,13] Mutations in Fpn lead to iron overload disease even though hepcidin levels are either normal or higher than normal. The clinical findings of Fpn disease are variable. The first report of Fpn disease was in a large pedigree in which a number of probands had high levels of serum ferritin, a hallmark of iron overload disease, yet serum transferrin levels were low.[14] Two other notable features were that iron loading of Küpffer cells occurred before iron loading of hepato-

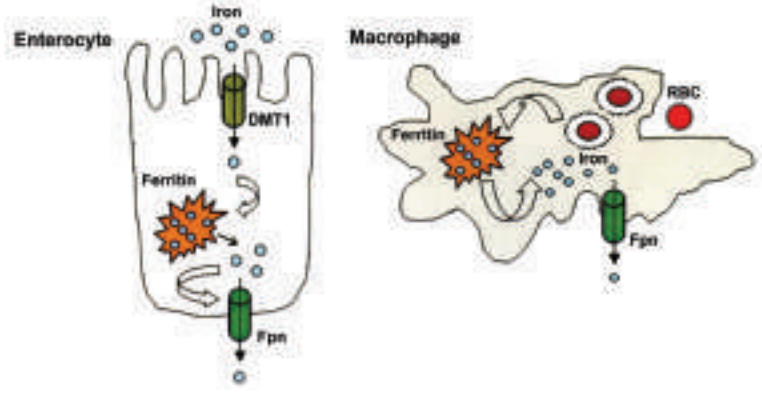

Figure 2. Schematic diagram of the location of ferroportin iron export in the enterocyte and the macrophage. Iron is transported into the enterocyte by DMT1 localized on the apical membrane. Within enterocytes, iron can be stored in ferritin or it can be transported out of the enterocyte by Fpn at the basolateral surface. Macrophages phagocytose damaged or senescent red blood cells. The hemoglobin is degraded within lysosomes and iron is released. The released iron can be stored in ferritin or it can be exported out of the macrophage by Fpn.

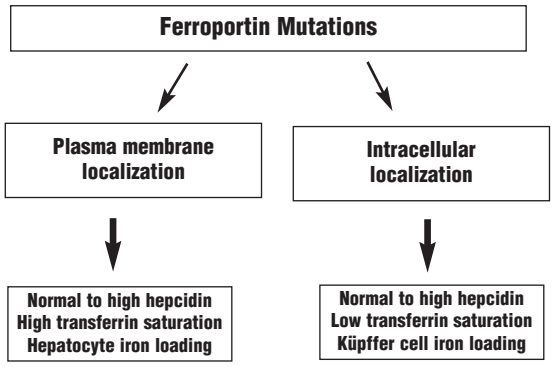

Figure 3. Effects of ferroportin mutations on iron metabolism.

cytes and that the inheritance was dominant. It was also observed that in some patients with Fpn disease, phlebotomy, the standard approach to clinical management of HH, led to dramatic decreases in transferrin saturation and anemia. Proof that mutations in Fpn are responsible for the disorder was confirmed by sequencing.[15] Since that initial report, subsequent studies reported similar case findings: a dominant form of iron loading disease in which Küpffer cells show early iron loading, high serum ferritin and low transferrin saturation. Cases of Fpn disease have been found worldwide and there is no evidence of a founder effect.

There are, however, reports of a dominant genetic iron loading disorder, which with the exception of the mode of transmission, shows a clinical phenotype indistinguishable from that of *traditional* HH with high transferrin saturation, hepatocyte iron loading and decreased iron in macrophages.[16] The genetic defect in this disorder was also shown to result from mutations in Fpn. Thus, mutations in Fpn result in a dominant genetic disorder with two different clinical manifestations, low transferrin saturation with Küpffer cell iron loading or high transferrin saturation with hepatocyte iron loading. The most notable feature about all of the Fpn mutations discovered to date is that they are all missense mutations; no nonsense mutations have been identified. Insight into the effect of Fpn mutations has come from studies in which mutations in Fpn were generated by site-specific mutagenesis and the mutant proteins expressed in tissue culture cells. Three different studies have utilized this approach and there is a consensus on the effect of mutations.[17-19] Fpn mutants can be placed in two categories. The first are mutations that result in an inability to transport iron. Most of the mutants that fit in this class result from a defect in the cell surface localization of Fpn. For Fpn to function it must be on the cell surface and many of the reported mutations result in a protein that is trapped in the endoplasmic reticulum and does not reach the cell surface. Fpn mutants that do not reach the cell surface are unable to export iron and consequently do not respond to hepcidin. The second class of Fpn mutants are those that are appropriately targeted to the cell surface and are capable of exporting iron but which do not respond to hepcidin. The mutants that have been studied to date seem to bind hepcidin but binding does not result in Fpn internalization and degradation. Thus, iron export from these mutants would be constitutive. The classification of Fpn mutants may provide an explanation for the variation in clinical phenotype with iron loading in Küpffer cells or iron loading in hepatocytes. Fpn mutants that are defective in iron export will lead to the symptoms of Fpn disease with Küpffer cell iron loading and low transferrin saturation. Mutations that affect Fpn localization to the cell surface should affect both intestinal iron transport and macrophage iron transport (Figure 3). Fpn patients are heterozygotes and contain both a mutant and a normal allele of Fpn. The normal allele might be sufficient to mediate intestinal iron transport (1-2 mg/day) but not sufficient to mediate macrophage iron transport (20-30 mg/day). Consequently, iron absorption from the gut may be normal but iron recycling from macrophages would be impaired, leading to low transferrin saturation and high levels of Küpffer cell iron loading. Patients with this class of Fpn mutations might be expected to respond poorly to phlebotomy therapy. Alternatively, Fpn mutants that are insensitive to hepcidin will result in constitutive iron export from both the intestine and macrophages. The increased plasma iron would exceed the ability of transferrin to bind iron resulting in iron accumulation in hepatocytes and phlebotomy therapy would be effective. Although hepatocytes have the ability to export iron, they also accumulate iron via the transferrin-independent iron transport system and the accumulation of iron would predominate under conditions in which plasma transferrin is saturated.

In genetic terms, the dominant inheritance of Fpn disease can be explained in two ways: either the mutant allele leads to haploinsufficiency or the mutant allele acts as a dominant negative. There are three lines of evidence that suggest that haploinsufficiency cannot explain dominant inheritance. First, no nonsense mutations have been identified in the Fpn gene. If inheritance was a result of haploinsufficiency such mutations should exist. Second, homozygosity for a targeted deletion in the Fpn gene in mice leads to early embryonic death.[20] Heterozygote mice, however, do not show evidence of iron-overload disease. The mice, if anything, show an iron-limited erythropoiesis when young. As the mice get older the anemia abates but no evidence of iron overload has been observed. The possible interpretations of these findings are either that deletion of the Fpn gene in mice is not a representative model of Fpn-linked iron overload in humans or that the human disease cannot be explained solely on the basis of haploinsufficiency. Third, De Domenico *et al.,* demonstrated that Fpn is a multimer, and that the mutant protein affects the behavior of the wild type protein.[19] Evidence support-

ing the multimeric structure of Fpn comes from several lines of experiments including co-immunoprecipitation studies in which immunoprecipitation of an epitope-tagged Fpn can also immunoprecipitate a different epitope-tagged Fpn. Furthermore, co-transfection experiments show that the expression of Fpn mutant proteins affects the subcellular localization and behavior of wild-type Fpn. Fpn mutant proteins that do not localize to the cell surface reduce the cell surface localization of wild type Fpn and Fpn mutant proteins that do not respond to hepcidin reduce the ability of wild-type Fpn to respond to hepcidin. If the Fpn multimer is a dimer then patients with a mutation in a single allele of Fpn would express three forms of Fpn protein: a wild-type homodimer, a mutant homodimer and a heterodimer composed of wild-type and mutant proteins. If the Fpn mutant protein does not get to the cell surface then only 25% of expressed Fpn proteins would be functional. In a model of haploinsufficiency 50% would be functional. Therefore, a dominant negative protein would result in a more severe effect on iron export than would haploinsufficiency. The behavior of the Fpn mutants affecting wild-type Fpn explains the genetics and most patients' phenotypes. One implication of these findings is that they can be used to rationalize clinical treatment. Patients with Fpn disease who show Küpffer cell iron loading and low transferrin saturation will not profit from phlebotomy therapy, while patients who have the *classical* HH phenotype of high transferrin saturation and hepatocyte iron loading will profit from phlebotomy. Unless the mutation is studied using *in vitro* approaches there is no way of predicting a patient's phenotype from the genotype. A more difficult problem to resolve is whether patients who have high transferrin saturation and high ferritin have the *classical* HH form of Fpn disease or whether these finding reflect later stages of Fpn disease. Such patients would require further study to determine the usefulness of phlebotomy therapy.

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