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A mutation in the *TMPRSS6* gene, encoding a transmembrane serine protease that suppresses hepcidin production, in familial iron deficiency anemia refractory to oral iron

Maria Antonietta Melis,[1] Milena Cau,[1] Rita Congiu,[1] Gabriella Sole,[2] Susanna Barella,[3] Antonio Cao,[2] Mark Westerman,[4] Mario Cazzola[5] and Renzo Galanello[1,3]

[1]Dipartimento di Scienze Biomediche e Biotecnologie, Università di Cagliari; [2]Istituto di Neurogenetica e Neurofarmacologia, CNR, Cagliari, and [3]Ospedale Regionale Microcitemie, Clinica Pediatrica 2, Cagliari, Italy; [4]Intrinsic LifeSciences, LLC, La Jolla, California 92037, United States and [5]Department of Hematology, University of Pavia and Fondazione IRCCS Policlinico San Matteo, Pavia, Italy

ABSTRACT

Background

Hepcidin plays a key role in body iron metabolism by preventing the release of iron from macrophages and intestinal cells. Defective hepcidin synthesis causes iron loading, while overproduction results in defective reticuloendothelial iron release and iron absorption.

Design and Methods

We studied a Sardinian family in which microcytic anemia due to defective iron absorption and utilization is inherited as a recessive character. Five members showed iron deficiency anemia that was not responsive to oral iron and only partially responsive to parenteral iron administration. To investigate the involvement of known genes implicated in iron metabolism we carried out linkage analysis with microsatellite markers mapping close to these genes. Afterwards, a genome-wide search was performed.

Results

No linkage was found between the phenotype of the patients and several known human genes involved in iron metabolism (*DMT1, TF, TFRC, ZIRTL, HAMP, HJV*). Genome-wide scanning by microsatellites and single nucleotide polymorphisms showed a multipoint LOD score of 5.6 on chromosome 22q12.3-13.1, where the matriptase-2 (also known as transmembrane protease, serine 6 or *TMPRSS6*) gene is located. Its murine counterpart (*Tmprss6*) has recently been found to be an essential component of a pathway that detects iron deficiency and suppresses hepcidin production. Sequencing analysis of *TMPRSS6* revealed a homozygous causal mutation, predicting a splicing error and a truncated TMPRSS6 protein in affected members. Homozygous subjects had inappropriately elevated levels of serum and urinary hepcidin.

Conclusions

The findings of this study suggest that the observed *TMPRSS6* mutation leads to overproduction of hepcidin and, in turn, to defective iron absorption and utilization. More generally, they confirm in humans the inhibitory effect of matriptase-2 on hepcidin synthesis already demonstrated in mice.

Key words: anemia, hepcidin, iron deficiency, matriptase-2, serine protease, *TMPRSS6*.

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Correspondence:
Renzo Galanello, MD, Clinica Pediatrica 2, Ospedale Regionale Microcitemie, Università di Cagliari, via Jenner s.n., 09121 Cagliari, Italy
E-mail: renzo.galanello@mcweb.unica.it

Introduction

The uniquely limited capacity of iron absorption and excretion makes maintenance of iron balance precarious in humans.[1] Iron deficiency affects more than half a billion people worldwide and is generally caused by acquired factors. It results from any condition in which dietary iron intake does not meet the body's demands, and pathological blood loss frequently contributes to this negative iron balance.

Body iron metabolism is regulated by several genes and a key role is played by hepcidin, which controls iron absorption and recycling.[2,3] Genetic iron overload is a relatively common condition, and may result from mutations of different genes of iron metabolism, generally resulting in suppression of hepcidin production.[4,5] By contrast, genetic iron-deficient anemia is seldom observed in humans, and the best characterized conditions involve a combination of iron-deficient erythropoiesis and parenchymal tissue iron overload. Atransferrinemia, also called familial hypotransferrinemia (OMIM #209300), is due to mutations in the transferrin gene (TF) that lead to low or undetectable levels of the carrier protein.[6,7] Mutations in the divalent metal transporter 1 gene (*DMT1*) have been found in patients with microcytic anemia, low serum ferritin, and liver iron overload (OMIM #209300).[8-10]

Previous studies have described a familial syndrome characterized by iron malabsorption, hypoferremia, and microcytic anemia that did not respond to oral iron and responded only partly to parenteral iron.[11-13] In this work, we studied a Sardinian family with this inherited condition. Genome-wide scanning allowed us to map its locus and to sequence a candidate gene. This led to the definition of a novel molecular basis of iron deficiency involving dysregulation of hepcidin production.

Design and Methods

Case report

We studied five patients, now aged 18-48 years, with iron-deficiency anemia that was not responsive to oral iron and only partially responsive to parenteral iron administration. The patients are members of four family units belonging to a large Sardinian pedigree that consists, in addition to the five affected individuals (two siblings, one double first cousin, and two second cousins), of nine unaffected individuals (Figure 1). Three of the patients (V-5, VI-1, VI-6) were referred to hospital between 8 and 12 months of age because of anemia, while the other two were found to be moderately anemic during the study of the family. Physical examination was normal in all patients except for the presence of moderate to severe pallor.

Hematologic studies in the five patients (Table 1) showed anemia of different severity, severe microcytosis [low mean corpuscular volume [MCV]), hypochromia [low mean corpuscular hemoglobin (MCH)], low serum iron, low transferrin saturation, and normal to increased serum ferritin. Known causes of hereditary microcytic anemia, such as congenital sideroblastic anemia and the hereditary microcytic anemia due to *DMT1* mutations, were excluded. β thalassemia was excluded in all patients by sequencing the β globin gene, while α globin gene DNA analysis showed, only in patient VI-6, the presence of an -α/-α genotype. Specific tests (antigliadin antibodies, anti-transglutaminase antibodies, stool test for occult blood, urinalysis) excluded other causes of iron-deficiency anemia, such as malabsorption and bleeding. Inflammatory conditions were excluded by measuring the levels of serum C-reactive protein, which were normal in all anemic patients.

Liver iron concentration, measured in two patients by magnetic resonance imaging, was in the normal range (0.43 and 1.1 mg/g dry tissue). Other consequences of iron deficiency, such as growth and developmental retardation and epithelial changes, were not detected in the patients.

Three of these patients were treated with oral ferrous sulfate without response; subsequent treatment with intravenous iron gluconate resulted in a partial response. Relevant red cell indices and other laboratory data for one of these patients (VI-6) before and after intravenous iron treatment are reported in Table 2, whereas the time course of hemoglobin, MCV and serum ferritin after repeated intravenous iron administrations is reported in Figure 2.

Anemia was more severe during childhood and the patients needed intermittent intravenous iron supply only during this period. As they became adults, they were able to maintain acceptable levels of hemoglobin (10.0–13.9 g/dL) albeit with typical findings of iron-deficiency erythropoiesis, i.e. reduced MCV (57–77 fL) and MCH (18.4–26.3 pg), low serum iron (13-31 μg/dL) and low transferrin saturation (4–9%) (Table 1). By contrast, serum ferritin levels tended to increase with age.

Biochemical analyses and iron status

Venous peripheral blood was obtained for biochemical blood tests and genetic analysis after written informed consent had been given by all the subjects. Red blood cell indices were determined by an electronic counter (Coulter LH750, Beckman Coulter, Fullerton, CA, USA). Other biochemical blood assays, including C-reactive protein, serum iron, ferritin, red blood cell zinc protoporphyrin and serum transferrin receptor, were performed using standard methods. Liver iron concentration was assessed using T2* measured with a gradient echo sequence, and also by measuring T2 using a spin echo sequence with a 1.5 magnet scanner (GE, Milwaukee, WI, USA).[14]

Linkage analysis

To investigate the involvement of known genes implicated in iron metabolism (*DMT1*, *TF*, transferrin receptor [*TFR*], zinc iron regulated transporter-like [*ZIRTL*], hepcidin [*HAMP*], hemojuvelin [*HJV*]), we carried out linkage analysis with microsatellite markers mapping close to these genes. Afterwards, a genome-wide search, excluding the sex chromosomes, was performed using the ABI PRISM Linkage Mapping set, at 10 cM

resolution index map. For the refined mapping of the candidate areas we examined additional microsatellite markers at a genetic distance of 1 cM, selected from the Genethon database. The genotyping data were processed using GENESCAN version 3.1, GENOTYPER version 2.5.

Linkage analysis was performed assuming a complete penetrant autosomal recessive trait, a disease frequency of 0.0001 and an identical recombination frequency for males and females. The marker alleles were considered of equal frequency except for markers that have already been tested in the Sardinian population during other genetic studies. Two-point linkage analysis was performed by the ILINK and MLINK routine of Fastlink package version 4.0p.[15] Support for linkage was obtained by multipoint linkage analysis using the LINKMAP routine of Fastlink. The genetic intermarker distances used in this analysis are the sex-averaged distances, based on published genetic maps (from the Marshfield Center for Medical Genetics) or on integrated maps (Unified Database, UDB). Haplotype analysis was performed using Cyrillic 2 (Cherwell Scientific). In case of uncertainty, GENEHUNTER version 2.0 16 was used to determine the most likely inference regarding the haplotypes of the individuals in the pedigree. In addition single nucleotide polymorphism (SNP)-based linkage analysis was performed in the candidate region identified by microsatellite linkage analysis. SNP were obtained from the public database of the National Center for Biotechnology Information, dbSNP. A total of 95 distinct SNP were genotyped in 14 family members.

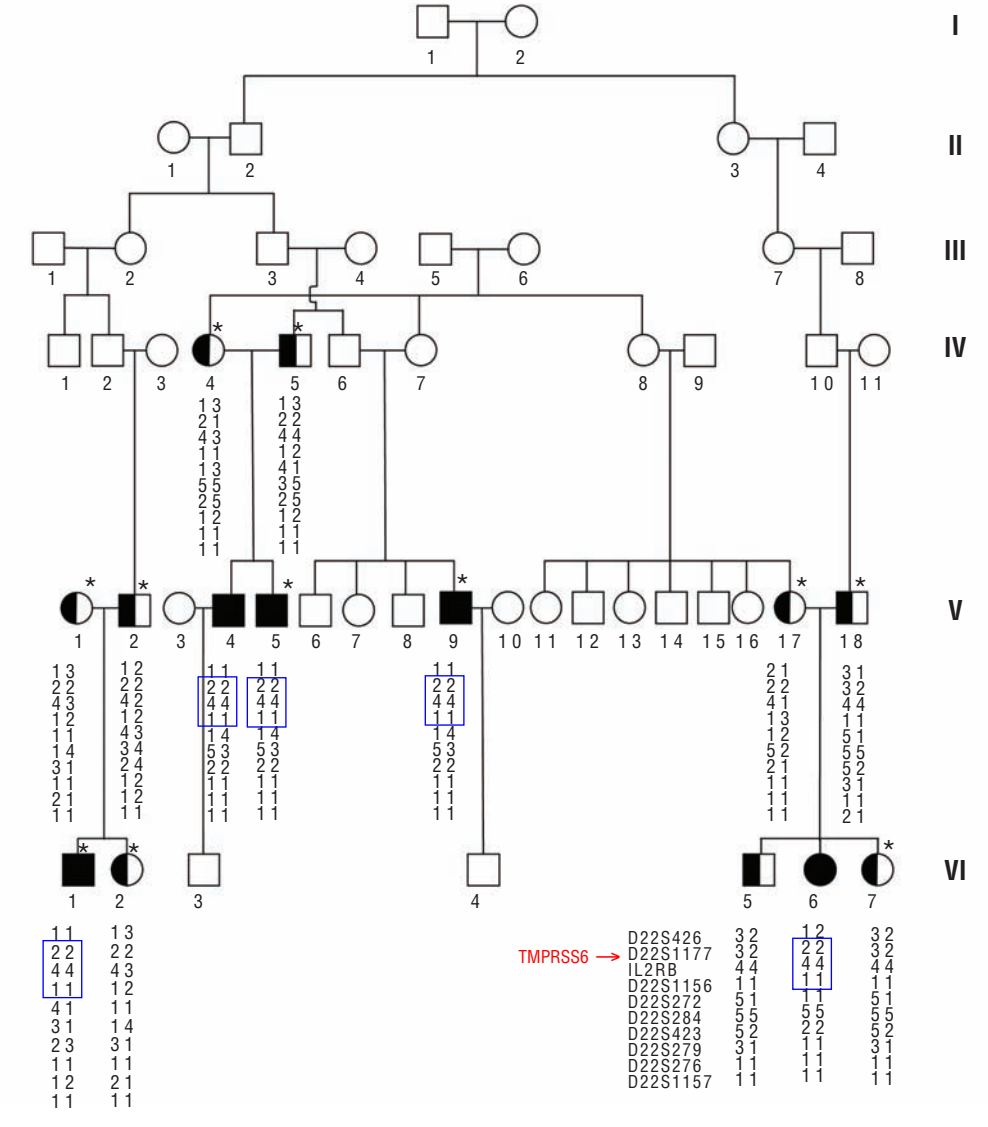

Figure 1. Family pedigree of the five subjects affected by hereditary iron-deficiency anemia. Pedigree of the family with informative haplotypes from selected markers of 22q12.3-q13.1. Blue rectangles demarcate the candidate gene region, which comprises only homozygous markers. The maximum LOD score was 5.6 near the marker D22S1177 where *TMPRSS6* resides (red arrow). Members who underwent genetic testing are indicated by an asterisk. Half-open symbols represent heterozygous carriers of the IVS6+1G->C *TMPRSS6* mutation and solid symbols represent homozygous carriers of the mutation.

Table 1. Main hematologic data, serum and urine hepcidin levels in patients homozygous for the *TMPRSS6* mutation.

	Family members				
	VI-1	*VI-6*	*V-4*	*V-9*	*V-5*
Age, year/sex	18/M	20/F	33/M	42/M	48/M
Hb, g/dL	10.0	9.3	10.0	12.8	13.9
MCV, fL	61	59	57	67	77
MCH, pg	19.6	19.1	18.4	22.1	26.3
Serum iron, µg/dL	16	16	13	31	30
Transferrin saturation, %	5	6	4	8	9
Serum ferritin, ng/mL	53	234	129	184	466
Serum hepcidin,* ng/mL	133.5	450.0	304.4	198.4	382.7
Urinary hepcidin,** ng/mg creatinine	2374	8649	1962	3468	Not done

**Reference range: normal individuals, 18-237 ng/mL; patients with iron deficiency anemia, < 5 ng/mL; **Reference range: 70 - 1762 ng/mg creatinine.*

Table 2. Red cell indices and other laboratory data in one patient before and after oral treatment with iron sulfate and before and after intravenous treatment with iron gluconate.

	Patient VI-6 (age: 11 years) Oral iron		*Patient VI-6 (age: 14 years) Intravenous iron*	
	Before treatment	*After treatment*	*Before treatment*	*After treatment*
Hb, g/dL	9.3	9.4	9.1	10.2
MCV, fL	55	54	54	58
MCH, pg	16.7	16.4	16.4	18.2
ZnPP,* mg/dL	155	167	155	112
Serum iron, µg/dL	36	28	16	31
Transferrin saturation, %	12	12	6	11
Serum ferritin, ng/mL	162	132	116	413

**ZnPP indicates red blood cell zinc protoporphyrin.*

DNA sequencing

Sequence analysis of the 18 exons and the intron-exon boundaries of the *TMPRSS6* gene was performed using an automated sequencer (ABI PRISM 3100, Applied Biosystem). Polymerase chain reactions were performed under standard conditions with primers designed according to the nucleotide sequences of *TMPRSS6* (*www.ensembl.org*).

Immunoassay for human serum hepcidin

Serum and urinary hepcidin levels were measured using a competitive enzyme-linked immunoassay (C-ELISA) for human hepcidin. In 24 normal subjects, serum hepcidin concentrations ranged from 18 to 237 ng/mL, while urinary hepcidin ranged from 70 to 1762 ng/mg creatinine.

Results

No linkage was found between the phenotype of the patients and several known human genes involved in iron metabolism (*DMT1, TF, TFRC, ZIRTL, HAMP, HJV*). Since the family originates from a small village in southern Sardinia, it was reasonable to suppose the existence of a common ancestor. The pedigree analysis suggested an autosomal recessive trait and was indeed consistent with a common ancestor. The genome-wide scan revealed that the region with the most evidence of linkage was on chromosome 22q12.3-13.1, with a maximum two-point LOD score of 2.99 for the marker D22S423. High-resolution mapping performed on chromosome 22q12.3-13.1 gave a multipoint LOD score of 5.6 near marker D22S1177. The linkage region spans about 7.2 cM between microsatellite markers D22S426 and D22S1177. No recombination was observed in the family but in this interval all affected individuals share two common regions of homozygosity.

To pinpoint the locus responsible for this hereditary microcytic anemia, linkage analysis was carried out using a total of 95 distinct SNP, which confirmed the presence of homozygosity regions but did not narrow the interval of interest. However, we noted that the *TMPRSS6* gene maps near marker D22S1177, where the maximum LOD score was obtained. The protein encoded by this gene was recently shown to be an essential suppressor of hepcidin gene expression, required for normal uptake of dietary iron in mice,[17,18] and was therefore considered a strong candidate gene.

Sequencing analysis of *TMPRSS6* revealed a homozygous G->C substitution at the +1 position of intron 6 (IVS6+1G->C), within the 5' splice donor site, in the

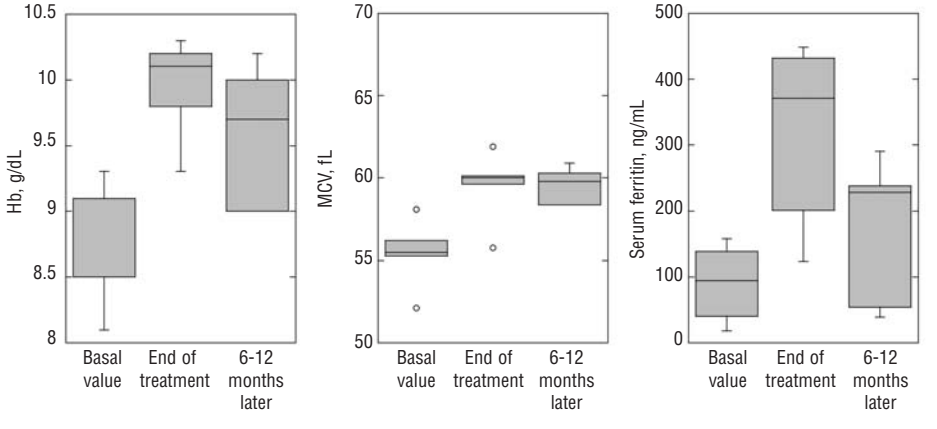

Figure 2. Time course of hemoglobin concentration, MCV and serum ferritin following administrations of intravenous iron. Values related to repeated administrations in two patients are shown in a box plot where circles indicate outliers.

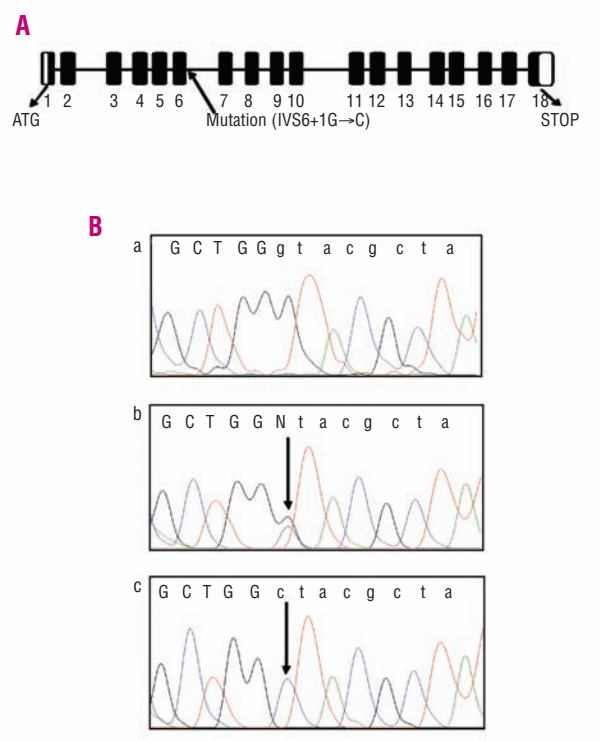

Figure 3. The IVS6+1G->C mutation of *TMPRSS6*. A. Schematic representation of the *TMPRSS6* gene. The arrow indicates the site of the mutation. B. Automated sequencing of genomic *TMPRSS6* DNA from: a) a normal individual; b) a subject heterozygous for the IVS6+1G->C *TMPRSS6* mutation; c) a patient homozygous for the IVS6+1G->C *TMPRSS6* mutation.

proband as well as in all the affected subjects (V-4, V-5, V-9, VI-1, VI-6) (Figure 3). The asymptomatic parents (IV-3, IV-4, V-1, V-2, V-17, V-18), and the unaffected sisters and brothers of the affected subjects (VI-2, VI-5, VI-7) were found to be heterozygous for the same mutation. The observed mutation predicts a splicing error and a truncated protein lacking the serine protease domain.[19]

Serum hepcidin concentration was measured in all five patients, and the results are reported in Table 1. It should be noted that normal serum hepcidin concentrations are two-fold higher in men than in women (median values 123 and 58 ng/mL, respectively), and undetectable (i.e., lower than 5 ng/mL) in patients with iron-deficiency anemia. Three out of five patients had serum hepcidin levels above the upper normal limit (i.e., 237 ng/mL). The remaining two patients had values in the normal range (133 and 198 ng/mL, respectively) despite iron-deficiency anemia, a condition in which hepcidin concentration is expected to be less than 5 ng/mL. All the four patients evaluated had increased urinary hepcidin/creatinine ratios above the normal range, including the two individuals with serum hepcidin in the normal range (Table 1). Serum hepcidin levels were in the normal range in four parents who had normal body iron status (*data not shown*).

Discussion

Systemic iron balance is basically maintained through the control of dietary iron absorption by duodenal enterocytes.[1] After entering the enterocytes, iron is transferred to plasma transferrin through ferroportin.[2] Iron absorbed through the gut does, however, account for only a part of circulating iron, most of which originates from the macrophages that recycle iron from senescent erythrocytes. In common with the process in enterocytes, iron is exported from macrophages to plasma through ferroportin. Hepcidin, a small peptide produced in the liver, blocks cellular iron efflux by binding to and inducing the degradation via internalization of ferroportin.[20] Hepcidin synthesis is regulated by iron, being increased by iron loading and suppressed by iron deficiency.[2] Additionally hepcidin production is decreased also by anemia and hypoxia, while is greatly increased during inflammation, primarily as a result of induction by interleukin 6.[2,5]

A recent study demonstrated that a membrane-bound serine protease encoded by the *TMPRSS6* gene is a physiological suppressor of hepcidin.[17,18] TMPRSS6 or matriptase-2 is a member of a family of cell surface proteolytic enzymes defined as type II transmembrane serine proteases.[19] The available evidence suggests that in the absence of *TMPRSS6*, increased hepcidin concentrations degrade intestinal ferroportin and interfere with normal iron absorption.[17,18] In fact, a splicing error in *Tmprss6* has been detected in *Mask* mice, which have a recessive chemically induced phenotype characterized by progressive loss of body hair and severe iron deficiency due to reduced absorption of iron from the gastrointestinal tract. *Mask* mice produce a truncated copy of the Tmprss6 protein lacking the serine protease domain and express inappropriately high levels of hepcidin. That these high levels can be responsible of iron deficiency and severe microcytic anemia was previously observed in transgenic mice over-expressing hepcidin.[21]

The human genetic ortholog *TMPRSS6*[19] is located in 22q12.3 where the linkage analysis performed in our family showed a multipoint LOD score of 5.6 and where all the affected subjects share a common region of homozygosity. These data, strongly suggestive of the presence of a locus for iron deficiency anemia in this region, prompted us to carry out sequencing analysis of the *TMPRSS6* gene, which revealed a homozygous splicing mutation in the patients. Like the *Tmrpss6* mutation in Mask mice, this splicing mutation predicts the deletion of the trypsin-like serine protease domain located at the carboxy terminal in the human gene.[19] Matriptase-2 mRNA is abundantly expressed in the liver of both human and rodents, and this suggests that the encoded protein functions in hepatocytes, where hepcidin is produced.[19] As in *Mask* mice, a truncated matriptase-2 protein lacking its protease function would involve inappropriately elevated

hepcidin production, which was indeed observed in all homozygous patients (Table 1).

Hepcidin overproduction is expected to cause iron trapping in enterocytes and in macrophages.[2] Hypoferremia, a consequence of the reduced absorption and recycling of iron, diminishes the amount of iron available for hemoglobin synthesis and red cell production, thus resulting in iron deficiency anemia.[22] The normal to slightly increased ferritin levels, despite the iron-restricted erythropoiesis, reflect the sequestration of iron in macrophages and, possibly, hepatocytes.[23] Indeed, the hematologic phenotype of patients homozygous for the *TMPRSS6* mutation is not identical to that of patients with classical iron-deficiency anemia, which is characterized by low serum iron, increased transferrin saturation and low serum ferritin. Their phenotype is more similar to that of patients with hepatocellular adenoma over-expressing hepcidin[24,25] or of anemic children with systemic-onset juvenile chronic arthritis, a condition associated with excessive production of interleukin-6[23] and likely with overproduction of hepcidin. Similar to this latter, the anemia observed in patients with the *TMPRSS6* mutation is a hybrid between classical iron-deficiency anemia (low body iron, inadequate iron supply for erythropoiesis) and anemia of inflammation (reticuloendothelial iron block, inadequate iron supply for erythropoiesis). By contrast, in adult patients with anemia of inflammation and overproduction of hepcidin, body iron content is normal but the iron is partially redistributed to reticuloendothelial cells.[26]

The increased levels of hepcidin also explain why our patients did not respond to orally administered iron and only partially to intravenous iron treatment. Orally administered iron enters enterocytes, but its delivery to plasma transferrin is markedly reduced as a result of elevated hepcidin levels, and the metal is lost with the normal shedding of the short-lived enterocytes. Colloidal iron (iron gluconate) given intravenously is taken up by reticuloendothelial cells.[23,27] Once these cells are iron-loaded (as shown by the elevated serum ferritin levels following intravenous iron administration – see Figure 2), a portion is likely exported to plasma transferrin despite increased hepcidin levels, and becomes available for erythropoiesis leading to partial correction of anemia with a slight improvement in MCV (Figure 2). The time course of serum ferritin levels following intravenous iron administration in our patients is similar to that previously observed in patients with systemic-onset juvenile chronic arthritis.[23] In fact, the uptake of colloidal iron by reticuloendothelial cells results in increased serum ferritin. Then, as iron is slowly released to plasma transferrin, both macrophage iron load and serum ferritin concentration decreases (Figure 2).

The fact that the anemia in our patients became less severe in adulthood is likely a consequence of the greater availability for erythropoiesis of the limited amount of dietary iron, which in childhood is needed for body growth, in particular for expansion of the red cell mass.

The loss-of-function mutation identified in our patients with hereditary iron deficiency anemia confirms the suppressive effect of *TMPRSS6* on hepcidin production already demonstrated in *Mask* mice,[17,18] and more recently observed in mutant mice deficient in matriptase-2.[28] In addition, another recent study indicates that iron deficiency anemia refractory to oral iron therapy can be caused by various germline mutations in *TMPRSS6*.[29]

Proteolytic events at the cell surface regulate many important cellular processes.[30] Matriptase-2 appears to play a crucial role in body iron homeostasis, and polymorphisms or mutations of this gene may partly explain the variability in iron absorption in humans. In the future, *TMPRSS6* might also be a target for manipulation of hepcidin production in disorders of iron metabolism.

Authorship and Discosures

Renzo Galanello, Maria Antonietta Melis, Antonio Cao and Mario Cazzola conceived this study, collected and analyzed data, and wrote the manuscript; Milena Cau and Rita Congiu performed the genomic and molecular studies; Gabriella Sole did statistical analyses; Susanna Barella collected clinical data; Mark Westerman did hepcidin measurements.

Clinical, hematologic and a few molecular features of this family were reported by Mario Cazzola at the 40th Annual Meeting of the American Society of Hematology, Miami, in 1998 (*Galanello R, Cau M, Melis MA, Deidda F, Cao A, Cazzola M. Studies of Nramp2, transferrin receptor and transferrin genes as candidate genes for human hereditary microcytic anemia due to defective iron absorption and utilization. Blood 1998;92:Suppl 1:669a*). Genomic studies indicating that the locus for hereditary microcytic anemia was on chromosome 22q13 were reported by Maria Antonietta Melis at the European Human Genetics Conference, Nice, June 16-19, 2007 (*Melis MA, Cau M, Congiu R, Sole G, Cao A, Galanello R. Identification of a gene involved in hereditary microcytic anemia due to defective iron absorption in a Sardinian family*). That *TMPRSS6* was a strong candidate gene was deduced by Mario Cazzola on December 9, 2007, when Dr. Bruce Beutler presented his studies on *Mask* mice in the Plenary Scientific Session at the 49th ASH Annual Meeting in Atlanta. The *TMPRSS6* mutation was identified in Cagliari in January 2008, while hepcidin measurements were performed in La Jolla. This study was submitted within March 1st, 2008, as an abstract to the 13th Congress of the European Hematology Association [*Melis MA, Cau M, Congiu R, Sole G, Barella S, Cao A, Westerman M, Cazzola M, Galanello R. Mutation in TMPRSS6, a suppressor of hepcidin gene expression, in familial iron deficiency anemia (Abstract). Haematologica 2008; 93 (Suppl 1):192*], and presented in the Presidential Symposium on June 14, 2008.

The authors declare that they have no potential conflicts of interest.

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