Is the acronym IRIDA acceptable for slow responders to iron in the presence of *TMPRSS6* mutations?

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Iron refractory iron deficiency anemia (IRIDA) is a recently described autosomal recessive disorder caused by mutations in *TMPRSS6*, the gene encoding matriptase-2. Patients have inappropriately high levels of hepcidin. Hypochromic microcytic anemia refractory to oral iron and only partially responsive to parenteral iron is the hallmark of this disorder. We report six patients from three unrelated families with mutations in the *TMPRSS6* gene, with three of the four identified mutations being novel. Although response to oral iron in IRIDA patients has been reported rarely before, all of our five patients receiving oral iron and our one patient supplemented with vitamin C responded to therapy at least to some extent. We think that IRIDA should be considered in the differential diagnosis of patients with findings of iron deficiency anemia responding inadequately to oral iron, particularly in countries with a high rate of consanguineous marriages like Turkey.

Key words: iron deficiency anemia, response to oral iron, genetics.

Iron deficiency anemia (IDA) is usually caused by inadequate dietary intake or chronic blood loss. However, disorders of iron metabolism resulting in microcytic anemia may also be hereditary in nature. A form of familial IDA referred to as iron refractory IDA (IRIDA), with no response to oral iron and only incomplete response to intravenous (IV) iron administration, is one of these disorders, the underlying genetic defect of which has been elucidated recently[1,2].

In fact, IRIDA was first described clinically in 1981 by Buchanan et al.[3], with several other reports having followed thereafter until 2008, when the underlying genetic background of the disorder could be identified[1,2]. Mutations in the transmembrane protease serine 6 (*TMPRSS6*) gene mapped on 22q12-13 cause IRIDA, the mode of transmission being autosomal recessive. *TMPRSS6* encodes matriptase-2 (MT-2), a transmembrane serine protease of the type-two transmembrane serine protease (TTSP) family, which is mainly expressed in the liver. The role of MT-2 in iron regulation was first shown by the discovery of a homozygous mutation of *TMPRSS6* in mask mice having microcytic anemia[4]. *TMPRSS6* knockout mice have a similar phenotype[5]: these mice develop anemia, lose trunk hairs and show decreased iron absorption because of high hepcidin levels.

Matriptase-2 (MT-2) regulates hepcidin expression by indirect control of Hamp expression in hepatocytes. Hamp is the gene encoding hepcidin. Membrane hemojuvelin activates Hamp expression through the bone morphogenetic proteins (BMPs) and son of mothers against decapentaplegic (SMAD) proteins pathway. As MT-2 cleaves hemojuvelin, it downregulates hepcidin production[6]. Consequently, MT-2 deficiency leads to inappropriately high levels of hepcidin. The 25-amino acid hepatic peptide hepcidin is the key regulator of iron homeostasis. It induces internalization of ferroportin, an iron exporter expressed in enterocytes, macrophages and hepatocytes. High levels of hepcidin thus downregulate enteric absorption of iron and its mobilization from intracellular stores[7].

In their laboratory evaluation, IRIDA patients typically display hypochromic microcytic anemia with very low mean corpuscular erythrocyte volume, low serum iron, highly unsaturated transferrin values, and normal/high ferritin levels. In contrast to typical IDA, in which hepcidin levels are low/undetectable, they are found to be normal/high in IRIDA cases[1,8-11]. In this article, we report the hematologic data and responsiveness to oral and/or IV iron therapy of six IRIDA patients from three unrelated families with *TMPRSS6* mutations.

Materials and Methods

Informed consent of the parents and blood samples of all family members of the cases for genetic analysis were obtained in accordance with the Declaration of Helsinki.

Serum hepcidin levels were measured by surface enhanced laser desorption/ionization time-of-flight mass spectrometry (SELDI-TOF-MS) as previously described[12]. Genomic DNA was extracted from peripheral blood using the QIAamp DNA Blood Mini Kit (Qiagen). All exons, exon-intron boundaries and a varying amount of the 5' and 3' flanking were examined by polymerase chain reaction (PCR) using specific primers. The genomic sequence from GenBank accession numbers NC_000022.10 was used as a reference sequence. Detailed protocols and primer sequences are available on request. The amplified products were isolated by electrophoresis on 1% agarose gel and purified using the QIAamp purification kit (Qiagen, Valencia, CA). Direct sequencing was performed using a fluorescence-tagged dideoxy chain terminator method in an ABI 310 automated sequencer (Applied Biosystem, Foster City, CA), according to the manufacturer's instructions.

Results

Family A

Two siblings born to healthy first-cousin parents, one a seven-year-old male (A1) and the other a five-year-old female (A2) thought to have α-thalassemia, were admitted to us for possible stem cell (cord blood) transplantation, as their mother was pregnant in her 34th gestational week. They were both born at term with birth weights appropriate for gestational age. In A1 and A2, hypochromic microcytic

anemia was first detected at 1.5 years and 6 months of age, respectively. To correct iron deficiency, they were given several courses of oral ferrous glycine sulphate, each time without any response. In both siblings, no infection explaining the anemia was found, and occult gastrointestinal blood loss and gluten enteropathy were excluded. Hemoglobin (Hb) electrophoresis, osmotic fragility, α-thalassemia mutational analysis involving 21 mutations, serum ceruloplasmin, karyotype, and bone marrow examination results were all normal (no sideroblasts observed). A2 was given erythrocyte transfusion once when she was four years old. At their first admission to our clinic, Hb values of the two siblings were 8.2 and 8.7 g/dl, respectively, with mean corpuscular volume (MCV) values of 52 and 57 fl, respectively. They both had serum iron of 6 μg/dl and transferrin saturation of below 5%. Serum ferritin was in the lower normal range (23 and 47 ng/ml) (Table I). With their hematologic data and unresponsiveness to several courses of iron therapy in their history, IRIDA was suspected. Mutational analysis confirmed the diagnosis, showing a novel homozygous mutation in the *TMPRSS6* gene (p.Gln571Glnfs*13) in the two siblings, the parents being heterozygous carriers*.

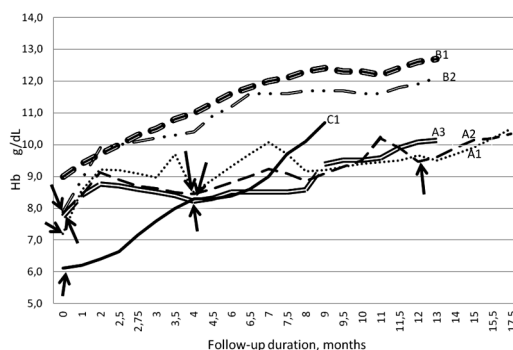The patients were given IV treatment (iron sucrose). In A1, Hb rose to a maximum of

Fig. 1. Changes in Hb of the patients over time (starting from first oral or IV treatment).

Circles, squares, triangles, and plus signs indicate times of IV iron administration in A1, A2, A3 and C1, respectively. B1 and B2 received no parenteral iron, and were followed up with oral iron solely. Due to the quite stable Hb levels for several months without therapy, IV iron was not readministered after the second course in A1 and A2, and oral iron was started after the 13th month of follow-up. Following two courses of IV treatment, C1 received oral iron supplemented with ascorbic acid after the 6th month.

9.2 g/dl and serum ferritin rose to 137 ng/ml. In A2, a similar response was observed, with Hb rising to a maximum of 9.1 g/dl and serum ferritin to 140 ng/ml. However, in both siblings, microcytosis persisted. Five months after IV treatment, a second course of IV iron was administered, which caused an increase in Hb from 8.5 to a maximum of 10.1 g/dl in A1 and from 8.4 to a maximum of 10.2 g/dl in A2 (Fig. 1).

In their follow-up for about eight months without therapy, Hb values of both A1 and A2 remained quite stable, with values between 9.0 and 10.0 g/dl, so we decided to begin oral iron (ferrous glycine sulphate; 6 mg/kg/d of elemental iron) to observe the response. They both responded partially, with their Hb values after four months of oral treatment increasing to 10.5 and 10.4 g/dl. However, microcytosis and low transferrin saturation persisted (Fig. 1).

In the mutational analysis of A3 done within her 4th month of life, homozygosity for the same IRIDA mutation as determined in her siblings was found. At that time, her Hb and MCV were 8.4 g/dl and 64 fl, and transferrin saturation was <5% (3.6%). She had serum ferritin of 365 ng/ml. At the age of eight months, she received IV iron. Her Hb rose from 7.8 g/dl to a maximum of 8.8 g/dl (Fig. 1). Ferritin rose from 89 to 441 ng/ml. However,

MCV remained consistently low (<55 fl). She received IV treatment again 10 months after the first parenteral iron course, with her Hb rising from 8.6 before treatment to a maximum of 10.1 g/dl (Fig. 1).

Family B

Two siblings born to healthy first-cousin parents, one a 10-year-old male (B1) and the other a seven-year-old female (B2), were admitted to our clinic due to IDA partially resolving after oral iron, and worsening again soon after cessation of the treatment. The 15-year-old brother was healthy, and the mother was pregnant in her 10th week of gestation. B1 and B2 were born at term with birth weights appropriate for gestational age. Hypochromic microcytic anemia was first detected at 2 and 5.5 years of age, with Hb values being 7.0 g/dl and 5.5 g/dl at that time, respectively. In both B1 and B2, occult blood loss and gluten enteropathy were excluded. Neither had an infection explaining anemia. Hb electrophoresis, α-thalassemia mutational analysis, serum ceruloplasmin, bone marrow aspiration, and biopsy results were all normal (no sideroblasts observed). At their admission, B1 and B2 had Hb of 9.0 and 7.8 g/dl, with MCV values of 59 and 57 fl, respectively. They had serum iron of 7 and 6 μg/dl. Transferrin saturation was low (<5%), and serum ferritin was in the

Table I. Data of the Cases, Including Hematologic Results at the Time of Admission and Current Values, Serum Hepcidin Levels at Diagnosis and Genotypic Findings

| Case | Age at first anemia presentation (years) | Data at first admission/current data | | | | | | Hepcidin (nmol/L)[a] | Genotype |
		Age (years)	Hb (g/dl)	MCV (fl)	MCHC (g/dl)	TSI (%)	Ferritin (ng/ml)		
A1	1.5	7.6/9.8	8.2/10.5	52/59	29.8/30.4	2/5	23/161	2.36	Homo.[b]
A2	0.5	5.8/8.0	8.7/10.4	57/66	31.4/31.6	<2/5	47/206	7.40	Homo.[b]
A3	0.3	[c]/2.0	7.8/10.1	52/57	30.3/30.4	3/5	89/228	7.76	Homo.[b]
B1	2.0	10.2/11.8	9.0/12.7	59/71	30.5/32.1	2/8	47/166	3.60	Homo.
B2	5.5	7.3/8.7	7.8/12.1	57/79	30.1/32.3	<2/15	50/262	15.88	Homo.
C1	0.5	3.5/4.4	6.1/10.7	48/65	30.1/31.4	<2/4	23/216	2.77	Comp. het.[d]

Hb: Hemoglobin. MCV: Mean corpuscular volume. TSI: Transferrin saturation index. MCHC: Mean corpuscular hemoglobin concentration. Homo.: Homozygous. Comp. het.: Compound heterozygous.
a Reference range for serum hepcidin: 3-7 nmol/L (n=57 normal individuals, median: 4.7 nmol/L), values in iron deficiency anemia are 0.04-0.12 nmol/L[21].
b Novel mutation.
c At the first admission, the mother was pregnant with the case. Hematologic data when she was 8 months old are shown.
d Both mutations are novel.

lower normal range (47 and 50 ng/ml) (Table I). Genetic analysis of the *TMPRSS6* gene revealed homozygosity of the two siblings for a previously reported mutation (p.Arg599Stop)[13], with the healthy brother being normal and the parents heterozygous carriers* (the newborn sister was found to be heterozygous later; she is one year old currently, and has normal Hb and MCV for her age).

It was decided to start oral iron (ferrous glycine sulphate; 6 mg/kg/d of elemental iron) instead of IV treatment in B1 and B2 owing to their history of partial responsiveness to oral therapy. With continuous 16-month treatment, their Hb rose progressively to 12.8 and 12.1 g/dl (Fig. 1), ferritin to 182 and 266 ng/ml, and MCV to 71 and 79 fl, respectively. The dose of iron was thereafter tapered to 3 mg/kg/d of elemental iron, and at their last visit, it was discontinued.

Family C

The 3.5-year-old male patient (C1) was born to healthy nonconsanguineous parents. He was born at term with a birth weight of 3150 g. The patient was admitted to our clinic due to microcytic anemia responding inadequately to iron treatment. His anemia was first detected at six months of age in a routine examination, with Hb of 6.8 g/dl and MCV of 59 fl. Following iron treatment for three months, his Hb rose to 8.1 g/dl. He received several courses of oral iron, after which his Hb reached values between 8.0 and 9.0 g/dl each time. He had no evidence of infection apart from urinary tract infection at one month of age, which did not recur. Occult blood loss and gluten enteropathy were excluded, and Hb electrophoresis, serum ceruloplasmin, bone marrow aspiration, and biopsy results were normal (no sideroblasts observed). At his first admission to us, his Hb and MCV were 6.1 g/dl and 48 fl, respectively. Serum iron was 6 μg/dl, with transferrin saturation <5%. Ferritin was in the lower normal range (23 ng/ml) (Table I). Genetic analysis confirmed IRIDA diagnosis, with compound heterozygosity, with both of the two mutations being novel (IVS10 +1 G>A; p.Cys510>Arg). Each of the parents was heterozygous for one of the two mutations*.

The patient was given IV iron, after which his Hb rose to a maximum of 8.3 g/dl. Being thought a partial-responder, IV iron was readministered two months later, after which his Hb remained almost the same (8.4 g/dl). He was then given oral iron (ferrous glycine sulphate; 6 mg/kg/d of elemental iron) supplemented with 100 mg oral vitamin C daily. After three months of this therapy, his Hb rose to 10.7 g/dl (Fig. 1). The dose of oral iron was then tapered to 3 mg/kg/d (oral vitamin C continued).

Since their last visits, all the cases remain well, growing satisfactorily. A1, A2 and C1 are receiving oral iron (C1 supplemented with ascorbic acid), whereas A3 is being followed closely with IV iron administration whenever thought indicated, as her compliance to oral iron was poor due to vomiting. B1 and B2 are off treatment.

Discussion

Iron refractory iron deficiency anemia (IRIDA) is a recently recognized inherited disorder of iron homeostasis causing hypochromic microcytic anemia. In fact, IRIDA is thought to be the most frequent among the 'atypical' microcytic anemias, which are rare inherited microcytic anemias different from the classic IDA or the classic form of X-linked sideroblastic anemia caused by mutations of the ALAS2 gene[14].

Hepcidin is the main regulator of iron homeostasis, and under normal conditions, the more severe the iron deficiency, the lower the hepcidin levels. However, factors other than iron status like inflammation and oxygen tension may influence hepcidin expression and production. In their research study, Bregman et al.[15] found baseline hepcidin levels of >20 ng/ml to predict nonresponsiveness to oral iron in patients with IDA. In IRIDA, mutations in the *TMPRSS6* gene encoding MT-2 result in inappropriately high hepcidin levels resulting in decreased iron absorption and turnover with no or inadequate response to oral iron.

With their hematologic data at admission, all of the six patients reported here fit perfectly with the diagnosis of IRIDA, with mild-moderate hypochromic anemia, remarkable microcytosis that was disproportionate to the degree of anemia, extremely low transferrin saturation and ferritin values in the normal range or at least not as low as one would

expect from transferrin saturation, and a history of inadequate response to oral iron (Table I). The remarkable increases in serum ferritin in all of the subjects who received IV iron (A1, A2, A3 and C1) also support the IRIDA diagnosis. In addition, all of the cases described here had either low normal (A1 and C1), normal (B1) or high (A2, A3 and B2) baseline serum hepcidin levels (Table I), which would be expected to be low/undetectable in classic IDA. This finding is also in accordance with the IRIDA diagnosis[1,8-11].

Few cases of IRIDA with response to oral iron have been reported before[16-19]. Notably, all of our patients receiving oral iron responded to therapy to some extent, though the response was not as marked, rapid or long-lasting as in classic IDA. The limited follow-up data of IRIDA patients in the literature point to a more severe anemia phenotype in childhood than in adulthood[2]. In adult patients, only mild anemia with persistently low transferrin saturation and remarkable microcytosis was reported before. In addition to utilization in erythropoiesis, iron is mandatory for several cellular metabolic steps, and the requirement is highest during early childhood owing to rapid growth, development and accompanying expansion of red cell mass. With increasing age, anemia may become less severe as a result of the greater availability of the limited amount of the dietary iron for erythropoiesis.

Among our patients, A1 and A2 showed a partial response to oral iron in spite of their history of unresponsiveness. This may be due to the tendency of the anemia to become less severe with advancing age. Similarly, in B1 and B2, acceptable Hb and even (almost) normal MCV values were achieved with 16-month continuous oral iron, which again may be explained by the older age of the patients in addition to the longer duration of the therapy (Fig. 1). The achievement of such responses in our subjects may be explained at least partially by their genotypes being different from those of cases reported so far, and the genotype-phenotype relationship in IRIDA patients needs further evaluation with larger series. Although the acronym IRIDA was proposed in 2008 by Finberg[1], the discrepancy in the response results might suggest a new description like sluggish responsive iron deficiency anemia

with mutations (SRIDAM), which also needs further assessment.

Cau et al.[17] reported a five-month-old female patient with IRIDA who was found homozygous for a *TMPRSS6* mutation. The infant did not respond to oral iron, and after showing a partial response to IV iron, she responded to a combination of oral iron and vitamin C. In C1, who had inadequate response to oral iron in his history and showed a less pronounced response to IV iron than other patients reported here, a combination of oral iron (ferrous glycine sulphate; 6 mg/kg/d of elemental iron) and ascorbic acid for three months resulted in an increase in the Hb level to as high as 10.7 g/dl. However, as the patient had not received oral iron of this dose for such a period before, we cannot conclude definitively that this response is attributable to combining oral iron with vitamin C. Though, as Cau et al. stated, if confirmed in more patients, this combination treatment may offer an alternative in the treatment of IRIDA patients and may simplify their management.

To our knowledge, five Turkish patients from four seemingly unrelated families have been reported so far in the literature[1,20]. Interestingly, all of them were homozygous for the same duplication leading to a frameshift and a premature stop codon (c.1904_1905dupGC, p.K636AfsX17). However, none of the families reported here had this mutation, and none of them shared a causative mutation. Altogether, four different mutations were identified in our subjects, three of them being novel. The nonsense mutation found in homozygous state in B1 and B2 was reported before in an English child with a clear-cut IRIDA phenotype[13]; however, the genotype was compound heterozygous in that case. The patient was reported to fail to respond to oral iron, which was given at 21 months of age for six months (200 mg/d), and at three and six years of age, each time for three months. Intravenous iron resulted in partial correction of anemia in that patient (Hb rose from 6.8 g/dl before treatment to a maximum of 9.8 g/dl after treatment). In contrast to the observations in that patient, in both B1 and B2, acceptable Hb values were obtained with 16-month full-dose oral iron therapy. This may be explained by the older age of our subjects at the beginning of the trial

of long-term iron therapy, the iron formula, dose and duration of the therapy, and the different genotype. B1 and B2 represent the first IRIDA cases in the literature with homozygous p.Arg599Stop mutation. Homozygosity for this mutation may be associated with less severe iron malabsorption than certain compound heterozygosities with combined residual functions, and some modifier genes enabling iron uptake by enterocytes or reducing hepcidin release by hepatocytes may play a role.

In cases with findings of IDA and poor response to oral iron, a possible diagnosis of IRIDA should be considered before performing more invasive procedures (like bone marrow examination), keeping in mind that this disorder is probably the most frequent cause of atypical microcytic anemias. Genetic analysis of the TMPRSS6 gene may help in the confirmation of the diagnosis. Due to its autosomal recessive mode of transmission, IRIDA may be more common in countries with a high rate of consanguineous marriages like Turkey.

* All of the mutations found were absent in 50 healthy controls and were not present in either the 1000 Genomes Project (http://browser.1000genomes.org/index.html) or the SNP (http://www.ncbi.nlm.nih.gov/SNP/) database.

REFERENCES

1. Finberg KE, Heeney MM, Campagna DR, et al. Mutations in TMPRSS6 cause iron-refractory iron deficiency anemia (IRIDA). Nat Genet 2008; 40: 569-571.

2. Melis MA, Cau M, Congiu R, et al. A mutation in the TMPRSS6 gene, encoding a transmembrane serine protease that suppresses hepcidin production, in familial iron deficiency anemia refractory to oral iron. Haematologica 2008; 93: 1473-1479.

3. Buchanan GR, Sheehan RG. Malabsorption and defective utilization of iron in three siblings. J Pediatr 1981; 98: 723-728.

4. Du X, She E, Gelbart T, et al. The serine protease TMPRSS6 is required to sense iron deficiency. Science 2008; 320: 1088-1092.

5. Folgueras AR, de Lara FM, Pendas AM, et al. Membrane-bound serine protease matriptase-2 (Tmprss6) is an essential regulator of iron homeostasis. Blood 2008; 112: 2539-2545.

6. Silvestri L, Guillem F, Pagani A, et al. Molecular mechanisms of the defective hepcidin inhibition in TMPRSS6 mutations associated with iron-refractory iron deficiency anemia. Blood 2009; 113: 5605-5608.

7. Nemeth E, Ganz T. The role of hepcidin in iron metabolism. Acta Haematol 2009; 22: 78-86.

8. Andrews NC. Forging a field: the golden age of iron biology. Blood 2008; 112: 219-230.

9. Brissot P, Bardou-Jacquet E, Jouanolle AM, Loreal O. Iron disorders of genetic origin: a changing world. Trends Mol Med 2011; 17: 707-713.

10. Camaschella C, Poggiali E. Inherited disorders of iron metabolism. Current Opin Pediatr 2011; 23: 14-20.

11. Tchou I, Diepold M, Pilotto PA, Swinkels D, Neerman-Arbez M, Beris P. Haematologic data, iron parameters and molecular findings in two new cases of iron-refractory iron deficiency anaemia. Eur J Haematol 2009; 83: 595-602.

12. Girelli D, Trombini P, Busti F, et al. A time course of hepcidin response to iron challenge in patients with HFE and TFR2 hemochromatosis. Haematologica 2011; 96: 500-506.

13. Guillem F, Lawson S, Kannengiesser C, Westerman M, Beaumont C, Grandchamp B. Two nonsense mutations in the TMPRSS6 gene in a patient with microcytic anemia and iron deficiency. Blood 2008 ; 112: 2089-2091.

14. Camaschella C. How I manage patients with atypical microcytic anaemia. Br J Haematol 2013; 160: 12-24.

15. Bregman DB, Morris D, Koch TA, He A, Goodnough LT. Hepcidin levels predict nonresponsiveness to oral iron therapy in patients with iron deficiency anemia. Am J Hematol 2013; 88: 97-101.

16. Khuong-Quang DA, Schwartzentruber J, Westerman M, et al. Iron refractory iron deficiency anemia: presentation with hyperferritinemia and response to oral iron therapy. Pediatrics 2013; 131: 620-625.

17. Cau M, Galanello R, Giagu N, Melis MA. Responsiveness to oral iron and ascorbic acid in a patient with IRIDA. Blood Cells Mol Dis 2012; 48: 121-123.

18. Beutler E, Van Geet C, te Loo DM, et al. Polymorphisms and mutations of human TMPRSS6 in iron deficiency anemia. Blood Cells Mol Dis 2010; 44: 16-21.

19. Guillem F, Kannengiesser C, Oudin C, et al. Inactive matriptase-2 mutants found in IRIDA patients still repress hepcidin in a transfection assay despite having lost their serine protease activity. Hum Mutat 2012; 33: 1388-1396.

20. Lehmberg K, Grosse R, Muckenthaler MU, et al. Administration of recombinant erythropoietin alone does not improve the phenotype in iron refractory iron deficiency anemia patients. Ann Hematol 2013; 92: 387-394.

21. De Falco L, Totaro F, Nai A, et al. Novel TMPRSS6 mutations associated with iron-refractory iron deficiency anemia (IRIDA). Hum Mutat 2010; 31: 1390-1405.