A child with severe iron-deficiency anemia and a complex TMPRSS6 genotype

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ABSTRACT

Objectives: We report a case of a 7-year-old girl with severe hypochromic microcytic anemia, who was unresponsive to classical iron supplements. We suspected IRIDA, iron-refractory iron-deficiency anemia, a genetic iron metabolism disorder, caused by TMPRSS6 variations. TMPRSS6 encodes matriptase-2, a negative regulator of hepcidin, and its pathological variants are related to normal to high levels of hepcidin. We analyzed the TMPRSS6 gene and we improved clinical management of the patient, selecting the appropriate supplementation therapy.

Intervention & Technique: The parenteral iron therapy was started, but the patient was only partially responsive and the anemia persisted. To confirm the diagnosis, the TMPRSS6 gene sequence was analyzed by DNA sequencing and other relevant biochemical parameters were evaluated.

Results: The TMPRSS6 sequence analysis showed a complex genotype with a rare heterozygous missense variant, in addition to other common polymorphisms. The serum hepcidin value was normal. We unexpectedly observed a normalization of patient’s hemoglobin (Hb) levels only after liposomal iron treatment.

Discussion and Conclusion: The proband was symptomatic for IRIDA during a critical phase of growth and development, but we did not find a clearly causative genotype. A long-term result, improving stably patient’s Hb levels, was obtained only after liposomal iron supplementation. Children may be at greater risk for iron deficiency and the degree of anemia as well as the response to the iron supplements varies markedly patient to patient. Here, we show the importance of comprehensive study of these patients in order to collect useful information about genotype–phenotype association of genes involved in iron metabolism.

Introduction

Iron-deficiency anemia (IDA) is a serious health problem worldwide. Low intake of dietary iron is the main cause of hypochromic, microcytic anemia; however, other conditions, such as bleeding, gastrointestinal malabsorption, or Helicobacter pylori infection, can also lead to iron deficiency and anemia [1,2]. Recently, a rare iron metabolism disorder was identified, named iron-refractory iron-deficiency anemia (IRIDA; OMIM #206200, ORPHA 209981). This disorder was first described in 1981 by Buchanan and Sheehan [3] in three siblings with IDA that was refractory to oral iron and only partially responsive to parental iron dextran, suggesting a possible genetic cause. Only 27 years later, Finberg et al. [4] demonstrated that this condition results from mutations in the TMPRSS6 gene, mapping to chromosome 22q12-q13, which encode matriptase-2 (MT-2). MT-2 contains a transmembrane domain, followed by a sea urchin sperm protein, enteropeptidase, and agrin (SEA) domain, a composite ectodomain with two complement factor C1r/C1s, urchin embryonic growth factor and bone morphogenetic protein (CUB) domains, three class A low-density lipoprotein receptor (LDLR) domains, and a C-terminal trypsin-like serine protease domain [5]. The liver is the primary site of MT-2 production, where it negatively regulates the synthesis of hepcidin, encoded by the HAMP gene, which is the main systemic iron regulatory hormone [4,6]. MT-2 cleaves hemojuvelin (HJV), a co-receptor for bone morphogenic protein, which is required for HAMP expression [7]. The reported causative mutations in TMPRSS6 are spread throughout the gene sequence, where they disrupt catalytic activity or protein–protein interactions, deregulating hepcidin production and causing its overexpression [8]. Considering the complexity of iron metabolism, combined with our limited knowledge, IRIDA due to a TMPRSS6 defect can be diagnosed only with certainty when the patient is homozygous or compound heterozygous for a pathogenic mutation [9]. The hematological parameters associated with IRIDA are hypochromic, microcytic anemia, with very low levels of serum iron and transferrin saturation (TSAT). Furthermore, serum ferritin levels are within the normal range, with a slight increase following intravenous treatment [10]. Another hallmark of IRIDA patients, as previously mentioned, is their inadequate response to oral iron treatment and
partial response to parental iron therapy. This genetic condition is probably underdiagnosed, and should be considered when other common causes of IDA have been ruled out. IRIDA patients have a wide geographic and ethnic distribution [11], with most being diagnosed as children, who despite having anemia, display normal growth, development, and intellectual performance [12]. The IRIDA phenotype is more evident in early childhood and adolescence, when high amounts of iron are needed for hemoglobin (Hb) synthesis, and becomes milder with aging [13], probably as a consequence of reduced iron requirement [10]. Here, we report the case of a young girl with an IRIDA-suggestive phenotype. To confirm the diagnosis, we analyzed the TMPRSS6 gene sequence and relevant biochemical parameters, and report the clinical follow-up under treatment.

Methods

We describe an Italian family with asymptomatic non-consanguineous parents and a daughter with unexplained microcytic anemia. To confirm the diagnosis of IRIDA, we looked for causative mutations in the TMPRSS6 gene by direct sequencing, in addition to measuring serum hepcidin and soluble transferrin receptor (sTfR) levels. The parents were also evaluated, but no iron-deficient anemia was evident.

Venous peripheral blood was obtained for biochemical blood tests and genetic analysis after parents had provided written informed consent. Genomic DNA was extracted from peripheral blood leukocytes with kit NLM AA1001 (Nuclear Laser Medicine, Settala, MI, Italy), according to the manufacturer’s instructions.

Polymerase chain reaction (PCR) primers were designed for the human gene TMPRSS6 (reference sequence NM_153609), to cover the promoter region and the sequence of the 18 exons, as well as the intron–exon boundaries. Detailed protocols and primer sequences are available on request.

The purified PCR fragments were sequenced with a BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, Austin, TX, USA) using an ABI PRISM 3100 automated sequencer (Applied Biosystems, Monza, Italy), according to the manufacturer’s instructions.

Serum hepcidin level was measured by competitive enzyme-linked immunoassay for human hepcidin (Hepcidin-25 bioactive ELISA, EIA-5258; DRG Diagnostics, Marburg, Germany). According to the information provided in the kit, the reference value for serum hepcidin is 10 ng/ml in healthy men and women. The serum level of sTfR was analyzed using an ELISA kit (Human Soluble Transferrin Receptor ELISA, EIA-4256; DRG Diagnostics, Marburg, Germany). The reported normal sTfR concentration ranges between 1.0 and 2.9 µg/ml in adults.

Case presentation

The proband was a 7-year-old girl that presented with pallor to the pediatric onco-hematology department of ‘G. Martino’ University Hospital of Messina, in May 2013. She had previously been diagnosed with nutritional IDA, for which she had been treated with oral iron therapy for 1 month, but without any follow-up management. At presentation, the laboratory parameters showed a severe microcytic, hypochromic anemia, with: Hb 4.4 g/dl, mean corpuscular volume (MCV) 56 fl, mean corpuscular hemoglobin (MCH) 14.7 pg, mean corpuscular hemoglobin concentration (MCHC) 26.1 g/dl, low serum iron 15 µg/dl, normal serum ferritin 48.2 ng/ml, and very low TSAT of 3%. The parents reported a healthy and balanced diet. The patient received a packed red blood cell transfusion. Thalassemia screening and genetic analysis for the common alpha and beta-globin gene mutations were tested, but results were negative for all family members. Acquired iron deficiency due to malabsorption (e.g. celiac disease) or other inflammatory or infective conditions were excluded, and there was no occult blood in the stool. At the hospital discharge, the Hb value was 8.9 g/dl; MCV was 66 fl; MCH was 19.3 pg; MCHC was 29.5 g/dl. After 2 months, the patient’s hematological data showed a reduction in Hb levels. Considering the patient’s previous partial response to oral iron therapy, she received intravenous iron gluconate (1.3 mg/kg/day) for 5 days. She partially responded to the parenteral iron treatment, with Hb and serum ferritin values increasing after treatment, as expected. The patient remained in good general condition, but recovery was partial and transient, and the Hb level reached 10 g/dl in October 2013, followed by a progressively decrease over the following months. Microcytosis and low serum iron also remained.

A second course of intravenous iron supplementation was administered 5 months after the first course. The response was similar, with no stable and adequate improvement in Hb level, and the anemia status of the patient persisted. The clinical conditions were highly suggestive of IRIDA syndrome, excluding other causes of anemia, also supported by the hematological data; therefore, we investigated the TMPRSS6 by direct sequencing, in addition to measuring the serum hepcidin level. The TMPRSS6 genotype of all family members is presented in Table 1.

The serum hepcidin concentration of the proband was 3.65 ng/ml, which was within the normal range. This would have been expected to be low/undetectable in classic IDA [14]. The sTfR level of the proband was increased to 3.2 µg/ml, similar to that observed in cases of both IDA and IRIDA.
In May 2014, we tested a different supplementation therapy in which the patient received liposomal oral iron (Sideral bimbi, PharmaNutra Spa) at a dose of 10 mg/day for 3 months. We observed a gradual increase in the patient’s Hb levels to be within the normal range, which had remained stable at the most recent follow-up appointment. The patient’s Hb and iron parameters under the different treatments are reported in Figure 1.

### Discussion

The prevalence of IRIDA is estimated to affect less than one person per million. Currently, up to 69 different mutations in the TMPRSS6 gene have been reported in 65 families [15]. Although these mutations are extremely rare, recent insights have revealed that TMPRSS6 polymorphisms may influence iron adsorption [16], contributing to the development of IRIDA symptoms [17].

The genotype of our IRIDA-like patient did not present severe TMPRSS6 mutations. However, the patient did present with the uncommon p.Arg446Trp variant (minor allele frequency 0.0036/18), and was homozygous for other common polymorphisms (p.Lys253Glu, p.Ser361=, p.Asp521 =, p.Val736Ala, p.Tyr739=). The latter two polymorphisms affect the catalytic domain. The patient did present other promoter and intronic variants of unknown clinical significance.

Several missense mutations in the CUB domain of the MT-2 protein, such as p.Arg446Trp, do not appear to affect the interaction of MT-2 with HJV. However, these mutations may induce modifications in RNA splicing and/or stability, protein activation and/or efficiency or alter the three-dimensional structure [11,18,19]. Jaspers and others reported the case of a girl with IRIDA who was heterozygous for the p.His369Asn variation in the CUB domain, suggesting allele haploinsufficiency. However, the only available data on the uncommon p.Arg446Trp variant suggest that this can produce a milder disease only when carried in combination with a severe mutation in TMPRSS6 [12].

The other polymorphisms identified in the coding sequence, particularly for p.Val736Ala, are associated with alterations in serum iron status, erythrocyte volume, and Hb levels, as confirmed by genome-wide association studies [13]. This is consistent with information from a recent Italian study, in which homozygosity for p.Val736Ala was frequently detected in anemic patients [16] and this variant is in linkage disequilibrium with p.Asp521 = [20]. Similarly, homozygosity for other common polymorphisms, such as p.Lys253Glu, p.Asp521 = and p.Tyr739=, has been reported to be present only in IDA patients and never having been detected in healthy controls [16].

### Table 1. TMPRSS6 genetic analysis.

| Exon | Nucleotide mutation | Amino acid change | SNP | Protein domain | Proband | Mother | Father |
|------|---------------------|-------------------|-----|----------------|---------|--------|--------|
| –    | c.-113T > C         | –                 |     |                | +/−     | +/−    | −/−    |
| –    | c.-120G > A         | –                 |     | Promoter       | +/−     | +/−    | −/−    |
| 1    | c.616 + 107T > C    | –                 |     | Intron         | +/+     | +/+    | −/+    |
| 2    | c.616 + 124C > T    | –                 |     | Intron         | +/+     | +/+    | −/+    |
| 3    | c.863 + 23A > G     | –                 |     | CUB1           | +/+     | +/+    | −/+    |
| 4    | c.2207T > C         | Tyr739=           |     | CUB1           | +/+     | +/+    | −/+    |
| 5    | c.432-71A > C       | –                 |     | Intron         | +/+     | +/+    | −/+    |
| 6    | c.432-71A > C       | –                 |     | LDLR2          | +/+     | +/+    | −/+    |
| 7    | c.432-71A > C       | –                 |     | LDLR3          | +/+     | +/+    | −/+    |
| 8    | c.1563C > T         | Asp521=           |     | Intron         | +/+     | +/+    | −/+    |
| 9    | c.1468 + 46C > T    | –                 |     | Intron         | +/+     | +/+    | −/+    |
| 10   | c.1468 + 46C > T    | –                 |     | Intron         | +/+     | +/+    | −/+    |
| 11   | c.1336C > T         | Arg446Trp         |     | Intron         | +/+     | +/+    | −/+    |
| 12   | c.1336C > T         | Arg446Trp         |     | Intron         | +/+     | +/+    | −/+    |
| 13   | c.1336C > T         | Arg446Trp         |     | Intron         | +/+     | +/+    | −/+    |
| 14   | c.1869-6_1869-2     | delCCCCA          |     | Intron         | +/−     | +/−    | −/−    |
| 15   | c.1869-6_1869-2     | delCCCCA          |     | Intron         | +/−     | +/−    | −/−    |
| 16   | c.1869-6_1869-2     | delCCCCA          | rs200434923 | Intron         | +/−     | +/−    | −/−    |
| 17   | c.2207T > C         | Val736Ala         |     | Intron         | +/+     | +/+    | −/+    |
| 18   | c.2217T > C         | Tyr739=           |     | Intron         | +/+     | +/+    | −/+    |

+/− = wild-type allele/wild-type allele.
+/- = mutated allele/wild-type allele.
+/+ = mutated allele/mutated allele.
+/- = wild-type allele/mutated allele.
−/− = mutated allele/wild-type allele.

TMPRSS6 variations identified in proband and in asymptomatic parents are reported.
Conclusion

For cases of IDA that are poorly responsive to oral iron, a possible diagnosis of IRIDA should be considered before performing more invasive procedures, such as bone marrow examination [2]. In the current study, we have shown that genetic analysis of TMPRSS6 is essential to confirm the clinical diagnosis, and may also be useful for predicting the patient’s response to iron treatment. Although genotype-phenotype studies in IRIDA patients are limited due to the relatively low number of patients, a correlation analysis by De Falco et al. [21] identified that patients carrying two nonsense mutations present with more severe anemia, microcytosis, and higher hepcidin levels. Sometimes, IDA is not completely refractory to treatment, as observed in our patient, who showed a partial, slow, and inadequate response to parental

Figure 1. The patient’s Hb trend and iron parameters during the clinical follow-up management.
iron therapy, but a surprisingly good response to liposomal iron. Liposomal iron is a new generation oral supplement with high gastro-intestinal absorption and bioavailability, as well as low incidence of side effects. Supplementation with liposomal iron has been shown to be safe and efficacious alternative to iron gluconate, previously used to correct anemia in non-dialysis chronic kidney disease [22].

For the first time, we report the potential benefits of treating a pediatric patient with persistent IDA with oral liposomal iron, who had previously demonstrated slight responsiveness to canonical oral or parenteral supplementation therapy. The use of liposomes as the iron carrier, associated with ascorbic acid, had a positive long-term effect on the patient’s Hb levels and iron status, with very good compliance and management.

Taken together, these data support the hypothesis that while co-inherited TMPRSS6 variants may not be able to induce a classic IRIDA phenotype, some haplotypes may increase susceptibility to iron deficiency-associated clinical symptoms, especially during childhood [17].

It is still unclear whether this can be explained by a combination of environmental factors and other factors associated with the polymorphisms, such as a low-expression allele, or whether the current Sanger sequencing strategy misses certain defects in the exons or introns of the gene, or in its regulatory regions [9]. Furthermore, we cannot exclude the presence of other gene defect, which may have contributed to the described phenotype.

Future studies in a large cohort of IDA patients will be necessary to identify polymorphisms responsible for a poor response to oral iron treatment [16], and to better understand their associations with iron status [23] in addition to identifying new interacting factors or mutated genes.

Genotype–phenotype correlation studies in a large number of IRIDA patients can be highly informative for patient management, in addition to increasing our knowledge on the function of TMPRSS6 during development, other factors involved in TMPRSS6 regulation, and its effects on body iron levels [23]. This could facilitate supplementation approaches to improve the iron status of anemic patients, which may involve a personalized medicine strategy in the future. Furthermore, iron fortification will be more cost effective with better patient compliance, and will be also associated with improved long-term outcomes.

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Disclosure statement
No potential conflict of interest was reported by the authors.

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