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Two novel mutations in *TpreMPRSS6* associated with iron-refractory iron deficiency anemia in a mother and child

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Abstract

In an iron deficient child, oral iron repeatedly failed to improve the condition. Whole exome sequencing identified one previously reported plus two novel mutation in the *TMPRSS6* gene, with no mutations in other iron-associated genes. We propose that these mutations result in a novel variety of iron-refractory iron deficiency anemia.

Keywords

Iron; Whole exome sequencing; Anemia

1. Introduction

The World Health Organization reports that iron deficiency is the most common and widespread nutritional disorder in the world, and estimates that 500 million to 2 billion people world-wide have iron deficiency anemia [1]. Oral iron therapy is the mainstay-treatment of iron deficiency, however, some children who are treated with oral iron have a poor response or no response [1]. This can be the case when children are not compliant with or are intolerant of the iron preparation [2]. In rare instances iron deficient children who are properly treated with oral iron fail to show any beneficial response. Genetically-based limitations in absorbing iron from dietary sources have been described, and are termed

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Conflict of interest

No author has a conflict of interest to report.

“iron-refractory iron deficiency anemia” (IRIDA). These typically have an autosomal recessive inheritance pattern due to compound heterozygous mutations in the *TMPRSS6* gene (TransMembrane PRotease Serine Six) with each mutation inherited from an asymptomatic parent [3,4].

We report a young girl with severe iron deficiency anemia where oral iron therapy repeatedly failed and intravenous iron administration was only somewhat effective in correcting her anemia. Using whole exome sequencing we found that she had one previously described mutation and two previously unreported mutations in *TMPRSS6* [5–8]. Her mother, who also has the phenotype of IRIDA, carries two of these mutations and her asymptomatic father carries the third. Evaluation of the mother showed that, like the child, she had very low serum iron, very low saturation of iron binding capacity, but high hepcidin levels. We propose that mother and child have a novel variety of IRIDA as a result of two *TMPRSS6* mutations, and that the child’s phenotype might be more severe than the mother’s on the bases of a third *TMPRSS6* mutation she inherited from her asymptomatic father.

2. Case

The patient was first evaluated in the hematology clinic at Primary Children’s Hospital for hypochromic microcytic anemia at the age of 20 months. She had been born following a normal pregnancy, labor, and term delivery to non-consanguineous Caucasian parents. Father was healthy with no history of anemia. Mother had albinism and nystagmus with poor vision and had anemia diagnosed during each of her pregnancies and was placed on oral iron during each. She had not received oral iron nor been evaluated for anemia in the preceding five years. The neonate did not have a hemoglobin (hgb) or hematocrit (hct) measured during the neonatal period.

At the age of six months she was seen by her pediatrician who diagnosed iron deficiency on the basis of a hgb of 8.0 g/dL, hct 26.3%, MCV 51 fL, MCH 15.5 pg, MCHC 30.3 g/dL, RDW 17.1%, WBC 12,800/ μ L, platelets 421,000/ μ L. Oral iron supplements were prescribed (2 mg/kg/day) with no significant improvement, thus her iron dose was increased to 6 mg/kg/day. She was seen by her pediatrician again at one year of age with a hgb of 5.2 g/dL, hct 20.2%, MCV 49 fL, MCH 12.6 pg, MCHC 25.8 g/dL, RDW 18.3%, serum iron 12 μ g/dL, TIBC 367 μ g/dL, transferrin saturation 3.2%, ferritin 15 ng/mL, reticulocytes 2.8%. It was thought that the family was non-compliant with the iron treatment, and she was given an intramuscular dose of iron dextran, 25 mg, and referred to the pediatric hematology clinic for further evaluation.

At her first pediatric hematology clinic visit (20 months of age) she was pale and behind on developmental milestones. She spoke only 2 or 3 words and was not walking well. Laboratory studies included; RBC 5.39×10^6 / μ L, hgb 8.5 g/dL, hct 31.5%, MCV 58.4 fL, MCH 15.8 pg, MCHC 27.0 g/dL, RDW 20.6%, platelets 436,000/ μ L, serum iron 8 μ g/dL, TIBC 276 μ g/dL (reference interval, 228–428), transferrin saturation 3%, free erythrocyte protoporphyrin 155 μ g/dL (reference interval, 0–35). Another dose of intramuscular iron dextran was given (50 mg). At the age of two years 10 months she developed what was

described as fainting spells, for which she received a packed red blood cell transfusion. The transfusion increased her hgb from 7.9 g/dL to 12.3 g/dL and alleviated her symptoms.

Over the next several years intravenous iron sucrose 50 mg was given seven times. This was associated with improvements in her laboratory values: hgb 11.3 g/dL, hct 35.6%, RDW 16.3%, MCV 66.7 fL, MCH 21.2 pg, MCHC 31.7 g/dL, serum iron 19 µg/dL, TIBC 229 µg/dL, transferrin saturation 8%. When she was 12 years-old, genomic DNA from her and both parents were evaluated at the University of Utah DNA Sequencing Core facility. Mother and child were found to have the same two mutations, in *cis*, in *TMPRSS6*. Iron studies were obtained on both, with similar findings (Table 1). The child and mother had evidence of biochemical iron deficiency (low serum iron and iron saturation), iron-limited erythropoiesis (low reticulocyte hemoglobin content and low MCV), borderline anemia, and both had elevated hepcidin levels (106.1 and 35.6 ng/mL; reference range < 2 ng/mL in iron deficiency). The father is healthy and has never been diagnosed with anemia or any hematological disorder.

3. Methods

The plasma level of hepcidin-25, measured by mass spectrometry (University of Utah Research Core Facilities), identifies human hepcidin-25 [9]. Samples were stored at -80°C then thawed and kept on ice prior to assay. To 100 µL of sample, 5 nM of human [$^{13}\text{C}_{18}$, $^{15}\text{N}_3$]-hepcidin ($\text{C}_{95}^{13}\text{C}_{18}\text{H}_{170}\text{N}_{31}^{15}\text{N}_3\text{O}_{31}\text{S}_9$, Peptide Institute Inc.) were added as internal standard (IS). 400 µL fetal bovine serum (FBS) and 250 µL 0.1% formic acid were added, and briefly vortex. A calibration curve of, 0.5 nM, 2 nM, 10 nM, 50 nM of human hepcidin ($\text{C}_{113}\text{H}_{170}\text{N}_{34}\text{O}_{31}\text{S}_9$, Peptide Institute Inc.) were spiked into 100 µL of FBS and treated along with patient samples in an identical manner, as follows.

Oasis HLB 1cm³ Solid Phase Extraction (SPE) column (Waters) was conditioned sequentially with 1 mL of methanol and 1 mL of water. The sample mixture was loaded on the conditioned HLB SPE column, and washed sequentially with: 1) 500 µL water, 2) 500 µL mixture of 30:75:5 (v:v:v) methanol: water: ammonium hydroxide($\text{NH}_3\cdot\text{H}_2\text{O}$), 3) 500 µL water. The hepcidin was eluted from the HLB SPE column by 500 µL of 90:10:0.1 (v:v:v) methanol: water: formic acid. The methanolic eluent was dried in a vacuum dryer. The dried sample was reconstituted with 50 µL of 20:80:1 (v:v:v) acetonitrile: water: formic acid, sonicated for 3 min and centrifuged at $20,000 \times g$ for 10 min. 10 µL of supernatant were injected into LC-MS for analysis.

Chromatographic separation was carried out using an Infinity 1290 LC system (Agilent). The column used for the analysis was a Sunfire C18 column (50×2.1 mm dimension, 1.8 µm particle size) (Waters). The mobile phases consisted of: 100% water with 1% formic acid (solvent A), 100% acetonitrile with 1% formic acid (solvent B). LC gradient used was: 0.0 min, 95% A, 5% B, flow rate 0.35 mL/min; 0.5 min, 95% A, 5% B, flow rate 0.35 mL/min; 3.5 min, 54% A, 46% B, flow rate 0.35 mL/min; 4.0 min, 0% A, 100% B, flow rate 0.35 mL/min; 6.6 min, 0% A, 100% B, flow rate 0.45 mL/min; 6.7 min, 95% A, 5% B, flow rate 0.35 mL/min; 9.0 min, 95% A, 5% B, flow rate 0.35 mL/min.

The MS/MS detection was carried out using an Agilent 6490 Triple Quad LC/MS system. The MS parameters used were: gas temperature 120 °C, drying gas flow 13 l/min, nebulizer pressure 40 psi, sheath gas temperature 300 °C, sheath gas flow 12 l/min, Vcap voltage 6000 V, nozzle voltage 3000 V, high pressure RF 190 V, low pressure RF 100 V. The following transitions were used for analysis: human hepcidin: 558.6 – > 693.3 for quantification and 558.6 – > 763.8 for qualification. The transition 563.1 – > 697.4 was used for quantification of human [¹³C₁₈, ¹⁵N₃]-hepcidin.

The obtained data were analyzed using MassHunter Quantitative Analysis program (Agilent). The ratios between peak areas of human hepcidin and [¹³C₁₈, ¹⁵N₃]-hepcidin in the calibration standard were plotted against the concentration to construct the calibration curve. The ratios found in the samples were used to calculate the hepcidin concentrations.

DNA from the patient and her parents was prepared from whole blood using the Qiagen genomic DNA extraction protocol (Qiagen, Valencia, CA) by the Translational Technologies and Resources (TTR) Core of the University of Utah Center for Clinical and Translational Sciences (CCTS). Whole exome sequencing was performed on the patient's DNA using Ion AmpliSeq Kit (Thermo Fisher Scientific, Grand Island, NY) at the University of Utah DNA Sequencing Core Facility. A variant call file was generated using the Ion Torrent Suite (Thermo Fisher Scientific, Grand Island, NY). The variant call file was annotated for functional gene consequences using ANNOVAR [10] and a scored list of candidate genes for an IRIDA phenotype was generated using Phenolyzer [11]. Variants were ranked according to functional consequence and relation to the IRIDA phenotype. Mutations were verified by PCR amplification of the *TMPRSS6* gene, submitted for Sanger Sequencing at the University of Utah DNA Sequencing Core Facility using published primers. The allelic genotypes of the parents were confirmed by PCR amplification and sequencing *TMPRSS6*.

Iron studies and CBCs were performed at Primary Children's Hospital Clinical Laboratories in accordance with Intermountain Healthcare Laboratory Services standard operating. The Sysmex quality control procedures were performed daily as recommended by the manufacturer.

4. Results

Plasma hepcidin levels, generally < 2.0 ng/mL in patients with iron deficiency anemia, were elevated in the child (106.1 ng/mL) and mother (35.6 ng/mL). Phenotype driven variant prioritization revealed three heterozygous damaging *TMPRSS6* variants in the child, a splice acceptor variant (IVS7 c.659del1), and two missense variants (A670V, c.2044C > T & V795I, c.2383 G > A, rs139105452). The splice acceptor variant and the A670V missense variant are not reported in the NHLBI GO Exome Sequencing Project [12]; the V795I variant is. It is a rare *TMPRSS6* variant (allele frequency 0.0095) and has been previously reported in cases of IRIDA [13]. Focused Sanger sequencing of DNA from both parents revealed the mother has the V795I and the splice acceptor variant and the asymptomatic father is heterozygous for the A670V missense variant. The A670V is next to one of the residues that forms the catalytic triad of the active site. The V795I is also within the catalytic region of the protein and is in the middle of a five residue stretch that is completely

conserved across 12 species from fish to man. Other genes involved in iron metabolism with no variations identified in the sequencing included *HJV*, *HFE*, *TF*, *TFR*, *DMT1*, *FPN1* and the *Steap* family of metalloredutases.

5. Discussion

Hepcidin, a peptide hormone synthesized primarily in the liver, binds to ferroportin, the transmembrane protein that exports iron from within cells [14]. The binding of hepcidin to ferroportin results in internalization and degradation of both molecules [15]. In this way hepcidin acts on enterocytes to block the release of dietary iron into the plasma, and it also acts on macrophages and hepatocytes to block recycling of stored iron [14,15].

Iron deficiency causes hepcidin levels to fall, which fosters an increase in dietary iron absorption, mobilization of stored iron, and acceleration of erythropoiesis previously restricted by iron limitation [20]. In our patient, however, and in others like her with IRIDA, inappropriately high hepcidin levels inhibit dietary iron absorption and impair the utilization of iron stores normally released by ferroportin. Thus, most patients with IRIDA have very low serum iron levels, very low transferrin saturations, microcytic anemia, and deficiencies in the iron-requiring enzymes relevant to neurodevelopment, cognition, and behavior.

Loss-of-function mutations in *TMPRSS6* are known to cause IRIDA. *TMPRSS6* encodes the protease matriptase-2, which regulates hepcidin levels. Damaging mutations in *TMPRSS6* lead to inadequate matriptase-2, resulting in an overabundance of hepcidin [7–9]. Different phenotypes of IRIDA have been reported. For instance, iron deficient siblings from Montreal were found to have compound heterozygous *TMPRSS6* mutations, resulting in p.G442F and p.E522K. Unlike our patient, and unlike most in the literature, those two children had hyperferritinemia and successful treatment with low-dose oral iron [4]. The common damaging *TMPRSS6* mutations result in hypoferritinemia and these patients typically have no beneficial response to oral iron [7–9].

Previously reported *TMPRSS6* mutations that are associated with IRIDA appear to have been inherited in an autosomal recessive manner; typically compound heterozygous mutations inherited *in trans* from asymptomatic parents. Our present case, however, is associated with two *TMPRSS6* variants inherited *in cis* from an affected mother to an affected child. The child's case might be complicated by inheritance of a third *TMPRSS6* variant from her asymptomatic father. It is unclear what role each of these *TMPRSS6* variants plays in the IRIDA phenotype of the mother and child.

Treatments that would lower the hepcidin level of patients with IRIDA should permit absorption of dietary iron, mobilization of iron stores, and thereby increase the availability of iron for erythropoiesis. Potential pharmacological approaches to lower hepcidin include anti-hepcidin antibodies, hepcidin sequestering agents, or various hepcidin inhibitors [16,17], however, none of these are currently available for clinical use. Erythropoiesis-stimulating agents are not effective in treating this condition [18]. While IV iron can have some benefit, iron administered IV is poorly utilized for erythropoiesis or incorporation into iron-containing enzymes. Most of the absorbed iron is stored in macrophages and

hepatocytes and remains trapped, unable to be exported because of high hepcidin levels and eventually resulting in iron toxicity. The long-term management of patients like ours should carefully attempt to weigh risks and benefits of intravenous iron supplementation and periodically assess iron overload of Kupffer cells and hepatocytes with liver function tests and abdominal MRI.

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Table 1

Clinical hematology laboratory test results, mother and child

	Serum iron (µg/dL)	Iron sat (%)	Transf (mg/dL)	Ferritin (ng/mL)	Reti (%)	Retic Hgb content(pg)	Hct (%)	Hgb (g/dL)	MCV (fL)	MCH (pg)	MCHC (g/dL)
Mother	18	7	210	136	1.4	25.8	38.9	12.0	79.6	24.5	30.6
Child	20	7	240	344	1.6	20.5	39.4	11.8	71.8	21.5	29.9
Ref. interval	37–170	20–50	212–360	10–204	0.5–1.5	29.1–35.5	36–46	12–16	80–100	26–34	32–36

Ref, reference (2.5th and 97.5th percentile reference intervals, Intermountain Healthcare Laboratories, Salt Lake City, UT, USA); Sat, saturation; Transf, transferrin; Retic, reticulocyte; Hgb, hemoglobin; Hct, hematocrit; MCV, mean corpuscular volume; MCH, mean corpuscular hemoglobin; MCHC, mean corpuscular hemoglobin concentration.