Two nonsense mutations in the TMPRSS6 gene in a patient with microcytic anemia and iron deficiency.

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**Key words**: iron, serine protease, microcytic anemia, hepcidin

**Section**: Red blood cells

**Short title**: Anemia and iron deficiency due to TMPRSS6 defect

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Abstract

Genetic causes of hypochromic microcytic anemia include thalassemias and some rare inherited diseases such as DMT1 deficiency. Here, we show that iron deficiency anemia with poor intestinal absorption and defective iron utilization of IV iron is caused by inherited mutations in TMPRSS6, a liver expressed gene which encodes a membrane-bound serine protease of previously unknown role that was recently reported to be a regulator of hepcidin expression.
Introduction
Hypochromic microcytic anemia is quite common in children with poor iron intake (1). Genetic causes include thalassemias (2) and some rare inherited forms with defective absorption and utilization of iron (3,4). Recently, several groups (5,6,7) including ours (7) have reported genetic defects of the iron transporter DMT1 in such patients. Noteworthy, DMT1 mutations causing microcytic anemia were associated with rather high serum iron and transferrin saturation as well as increased hepatic iron stores (8). Here we report the case of a young patient with microcytic iron-refractory iron deficiency anemia (IRIDA) who is a compound heterozygote for 2 nonsense mutations in the transmembrane serine protease TMPRSS6. Mutations in this gene have been recently described in the Mask mouse, a mouse model of IRIDA (9)(10) and TMPRSS6 knock-out mice also display the same phenotype (11). Our observation confirm a very recent communication reporting germline mutations of TMPRSS6 in IRIDA patients (12).

Patient, material and methods
The proband was born to non-consanguinous English parents and was 18 months old when microcytic hypochromic anemia was first diagnosed. He presented with rotavirus gastroenteritis and he was incidentally found to have hemoglobin concentration of 60 g/L, mean corpuscular volume of 47 fL and serum iron below 5 µmol/L. He is one of dizygotic twins and has one elder sister. In retrospect, he was thought to be mildly symptomatic of anemia as parents felt he was more lethargic than his twin sister. Both parents are normal and he is the only affected sibling (Table 1). Serum haptoglobin concentration was normal. Thereafter, serum iron was consistently low (<5 µmol/L), transferrin saturation was less than 5% and serum ferritin was usually in the lower normal range. Three attempts were undertaken to correct the iron deficiency with 200 mg oral iron per day for either six months when he was 21 month old or for three months at the age of three and once again at the age of six. He failed to respond to oral iron, with no improvement in hemoglobin levels and serum iron remaining below 5 µmol/L. At the age of seven, iron absorption was assessed by administration of oral ferrous iron at 3mg/kg followed by measurement of serum iron at 3 hours. Defective iron absorption was demonstrated by a rise of 13 µmol/L in serum iron as compared to a minimal expected rise of 18 µmol/L for children with comparable IDA (13). Consequently, he was given a course of intravenous iron (iron sucrose 100 mg weekly during 3 consecutive weeks). Serum ferritin rose from 11 µg/L to 109µg/L and hemoglobin rose from 68 g/L before the treatment to a maximum of 98 g/L. However microcytosis with pencil cells and hypochromia
persisted. At that time, bone marrow was mildly hypocellular with ragged erythroblasts, nuclear-cytoplasmic asynchrony, intercytoplasmic bridging, no sideroblast. Iron was detectable on trephine biopsy.

Since that time the patient has been off treatment and remains well, growing satisfactorily. Informed consent of the parents and blood samples of all family members were obtained for genetic analysis according to the Declaration of Helsinki. Genomic DNA was extracted and DMT1 gene was explored as previously described (7). Each of the TMPRSS6 18 exons and intron exon junctions was amplified by PCR with a set of primers complementary to flanking intron sequences (Supplemental Table). The PCR fragments were sequenced using a Big Dye terminator kit (Applied Biosystems, CA).

A new experimental ELISA was used to measure plasma hepcidin in frozen samples stored at -80°C. Duplicate 5μl samples were diluted 1:20 in PBS, and the refolded, bioactive form of hepcidin was measured using previously described polyclonal antibodies (14) in a competitive ELISA (Intrinsic LifeSciences, La Jolla, CA), using HPLC-purified, synthetic, bioactive hepcidin as a standard.

**Results and discussion**

In search of rare causes of genetic microcytic anemia, we first sequenced the DMT1 gene in a patient with IRIDA without finding any mutation. We then tested the TMPRSS6 gene because of the similarities in the hematological profile between our patient and the recently described Mask mouse (9)(10). This mouse strain presents iron deficiency and microcytic anemia related to the inability to repress hepcidin, a key regulator of iron metabolism. The Mask phenotype was ascribed to a homozygous loss of function mutation in the Tmprss6 gene, a gene mostly expressed in the liver, encoding a membrane-bound serine protease of previously unknown role (15,16).

In our patient, two nonsense mutations were identified by sequencing the TMPRSS6 gene: one heterozygous c.1179T>G substitution in exon 10 introducing a nonsense codon p.Y393X and a c.1795C>T substitution in exon 15 introducing another nonsense codon p.R599X (cDNA sequence reference AY055384) (Figure 1). These mutations are both predicted to delete the serine protease domain from the encoded protein unless the mRNA harbouring premature translation termination codon is rapidly degraded through the nonsense-mediated RNA decay surveillance pathway (17). Analysis of the parents’ DNA revealed that each parent is heterozygous for one of the nonsense mutations, confirming that each mutation in the proband
has targeted a different allele, thus resulting in the absence of normal gene product. The twin sister is heterozygous for the p.R599X while the elder sister does not carry any TMPRSS6 mutation (Figure 1). A very recent communication reports several cases of patients with TMPRSS6 mutations and inappropriately normal hepcidin level with regard to the degree of anemia (12). Hepcidin is a major regulator of iron homeostasis, which binds to ferroportin and induces its internalization and degradation. Therefore, high hepcidin levels prevent duodenal iron absorption as well as recycling of heme iron by macrophages (18). In our patient, plasma hepcidin levels were within the normal range established using a new experimental competitive ELISA, despite IDA (Table 1). Undetectable hepcidin levels have been found in 18 of 19 IDA patients (M Westerman; unpublished data) and were also shown by mass spectrometry to be unmeasurable in IDA patients (19). Thus, the proband had hepcidin levels that were inappropriately high relative to iron status. Several other observations are consistent with a defective hepcidin down-regulation. First, oral iron supplementation was not effective in correcting anemia. Second, absorption of a bolus of ferrous iron increased serum iron levels by 13 µmol/L rather than the predicted minimum of 18 µmol/L, suggesting a partial block in intestinal iron absorption (14). Finally, our patient had constantly low serum iron concentrations despite the presence of detectable iron stores in bone marrow after intravenous iron therapy and serum ferritin values within the normal range.

This patient presents distinctive features from the previously reported patients with DMT1 mutations (5,6,7). The anemia was detected at birth in DMT1 patients while here the diagnosis was made at 18 months, suggesting that iron transfer from the mother to the fetus is not altered by TMPRSS6 mutations. Similarly, in the paper by Finberg et al., none of the anemia was diagnosed at birth (11). Furthermore, DMT1 patients always had high serum iron values (8), in contrast with the strikingly low values found in our patient and they developed severe liver iron overload at a young age.

Sixteen different genes of the TMPRSS family are known in 2008 and mutations in TMPRSS1, 2, 3, 5 are apparently involved in non syndromic deafness (20) and in the pathogenesis of cancers (21,22). The role of TMPRSS6 in down-regulating hepcidin expression in response to IDA remains to be established.
Acknowledgments
This work was supported by an ANR-GIS Institut des Maladies Rares grant to Carole Beaumont (RPV07047HSA). The authors are grateful to Dr. Gordana Olbina (Intrinsic LifeSciences) for performing the hepcidin assays.

Authors’ contributions
FG and CK designed and performed sequencing experiments and analyzed data, SL diagnosed the patients, MW performed hepcidin assay, CB and BG designed the study, analyzed the data and wrote the paper.
The authors declare no conflict of interest.
References

Table 1: Biological parameters of the patient and of his relatives at the time of the present study (2008)

<table>
<thead>
<tr>
<th></th>
<th>Normal values</th>
<th>Patient</th>
<th>Father</th>
<th>Mother</th>
<th>Twin Sister</th>
<th>Elder Sister</th>
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<tbody>
<tr>
<td><strong>Age (years)</strong></td>
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<td></td>
<td></td>
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<tr>
<td>Hb (g/L)</td>
<td>120-155</td>
<td>86</td>
<td>151</td>
<td>127</td>
<td>144</td>
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<td>Hematocrit</td>
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<td>MCV (fL)</td>
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<td>85.7</td>
<td>85.8</td>
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<td>Reticulocytes (10⁹/L)</td>
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<td>30</td>
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<td>Serum Iron (µmol/L)</td>
<td>14.5-26.0</td>
<td>2.44</td>
<td>22</td>
<td>14.5</td>
<td>12</td>
<td>19</td>
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<td>Serum Ferritin (µg/L)</td>
<td>Males 30-300</td>
<td>9</td>
<td>88</td>
<td>10.4</td>
<td>9</td>
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<td>Females 20-150</td>
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<td>Soluble transferrin receptors (mg/L)</td>
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<td>1.52</td>
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<td>Plasma hepcidin (ng/mL)</td>
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<td>Females: 16-288*</td>
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</table>

*5-95 % range: T Ganz, G Olbina, Girelli, E Nemeth, M Westerman: unpublished data
ND: not determined
Legend to Figure 1

A- Pedigree of the family with the *TMPRSS6* genotypes
B- Sequencing traces from exon 10 and exon 15 of *TMPRSS6*. Arrows indicate heterozygous nucleotide substitutions leading to Y393X and R599X nonsense codons.
Figure 1

A

\[ \text{R599X/wt} \quad \text{Y393X/wt} \]

\[ \text{wt/wt} \quad \text{R599X/Y393X} \quad \text{R599X/wt} \]

B

\text{Y393X (exon 10)}

\text{normal}

\text{patient}

\text{R599X (exon 15)}
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