MICROCYTIC ANEMIA AND HEPATIC IRON OVERLOAD IN A CHILD WITH COMPOND HETEROZYGOUS MUTATIONS IN DMT1

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Running title: Clinical phenotype of DMT1 mutations.

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Abstract

Divalent metal transporter 1 (DMT1) mediates apical iron uptake in duodenal enterocytes and iron transfer from the transferrin receptor endosomal cycle into the cytosol in erythroid cells. Both mk mice and Belgrade rats, which carry an identical DMT1 mutation, exhibit severe microcytic anemia at birth, defective intestinal iron absorption and erythroid iron utilization. We report the hematological phenotype of a child, compound heterozygote for two DMT1 mutations, who was affected by severe anemia since birth and showed hepatic iron overload. The novel mutations were a 3 bp deletion in intron 4 (c.310 - 3_5del CTT) resulting in a splicing abnormality and a C>T transition at nucleotide 1246(p. R416C). A striking reduction of DMT1 protein in peripheral blood mononuclear cells was demonstrated by western blot. The proband required blood transfusions until erythropoietin treatment allowed transfusion-independence with haemoglobin levels between 7.5-9.5 g/dL. Hematological data of this patient at birth and in the first years of life strengthen the essential role of DMT1 in erythropoiesis. The early onset of iron overload indicates that, as in animal models, DMT1 is dispensable for liver iron uptake, whereas its deficiency in the gut is likely bypassed by upregulation of other pathways of iron absorption.
Introduction

Hypochromic microcytic anemia may result from inherited defects of globin chain synthesis or, more commonly, from acquired iron deficiency, usually due to low dietary iron intake, increased requests for growth or pregnancy, malabsorption or chronic blood loss. Genetic models of iron deficiency have been identified in animals. Autosomal recessive defects of iron absorption have been firstly characterized in rodents \(^1\text{-}^2\), and shown to be due to inactivation of the iron transporter Divalent Metal Transporter 1 (\textit{DMT1}) or \textit{Nramp2/DCT1}\(^3\), also called \textit{SLC11A2}. \textit{DMT1} is a transmembrane protein expressed at the brush border of duodenal enterocytes, where it is involved in dietary non-heme iron uptake from the intestinal lumen\(^4\). \textit{DMT1} also functions in the transferrin endosomal cycle of the erythroid precursors, where it transfers iron from the site of uptake through transferrin receptor, to cytosol and mitochondria for utilization\(^2\). A missense mutation of \textit{DMT1} gene that results in the substitution of arginine for glycine at position 185 of the protein (G185R) leads to severe iron deficiency at birth both in \textit{mk} mouse\(^1\) and Belgrade (\textit{b}) rat\(^2\). The mutation has multiple effects on folding, glycosylation, stability and transport activity, globally reducing the function of \textit{DMT1} protein\(^5\text{-}^7\).

Recently the first human model of the disease was reported in a young female who had a congenital defect of the erythroid pathway of iron utilization with resulting iron deficient erythrocytes, but, differently from animal models, had increased hepatic iron stores\(^8\). The patient carried an homozygous mutation of the last nucleotide of \textit{DMT1} exon 12, which caused a conservative amino acid change (E399D), but exaggerated the exon 12 skipping that occurs physiologically in minimal amounts\(^9\). In the proband, a shorter size RNA lacking exon 12, was the prevalent \textit{DMT1} variant both in reticulocytes and in other blood cells. In addition, \textit{DMT1} protein reduction was documented by cytofluorimetric studies in patient reticulocytes, as compared to normal controls\(^8\). Since abnormal \textit{DMT1} protein was not identified in the proband intestinal cells\(^9\) it was concluded that the defect was a quantitative reduction of the \textit{DMT1} RNA.

Here we report the case of a 5-yr-old child affected by severe, unexplained microcytic hypochromic anemia from birth, who was compound heterozygote for two novel \textit{DMT1} mutations. Liver iron overload was documented in early life. The severe degree of anemia was partially responsive to recombinant erythropoietin treatment.
Case report

The propositus was born at term by cesarian section from non-consanguineous parents living in Southern Italy. Body weight at birth was 2.530 kg. At physical examination the newborn was extremely pale with mild spleen and liver enlargement. Anemia was severe (Hb 4 g/dl) with remarkable microcytosis (MCV 71 fl) and hypochromia (MCH 13 pg). The newborn underwent transfusions of 1 unit of red blood cells (RBC) at birth and a second unit two days later (Table 1). At the age of two months the patient was referred to our Department for persistent anemia. He was pale with hepa-to-splenomegaly. Hematological evaluation showed: RBC 2.9 x10⁶/µL, Hb 7.4 g/dl; HCT 25 %; MCV 75 fl; MCH 14 pg. Reticulocytes 21x10³/µL; PLT 455 x10³/µL; WBC 6.1 x10³/µL with normal differential leucocyte count. Peripheral blood smear showed extreme anisocytosis and poikilocytosis. At three months of age the degree of anemia was stable, transferrin saturation reached 100% and serum ferritin was remarkably elevated (864 ng/ml - Table 1). Other blood tests including total and indirect bilirubin, LDH, Coomb’s test, B₁₂ and folate levels, Hb electrophoresis and karyotype were normal. Bone marrow aspirate showed remarkable erythroid hyperplasia. Free erythrocyte protoporphyrin (FEP) was increased (4,7 µg/gHb; normal values <3 µg/g Hb). Alpha/non alpha globin chain synthetic ratio of peripheral blood reticulocytes was 0.85 (normal value: 1.0±010).

Due to the presence of mild neutropenia (820/µL), slight increase of liver enzyme levels (GOP, GPT and γ-GT), screening for viral infections was carried out that revealed positive anti-CMV Ig M antibodies, positive CMV PCR tests, positive pp65 Ag and anti-neutrophil antibodies. A cycle of Gancyclovir treatment was performed. Three months later tests for CMV infection, including PCR, were negative but anemia did not reverse.

Two further blood units were administered at the age of 2 and 3 months for severe anemia (Table 1). At this time administration of recombinant erythropoietin (rEpo) was started at the dose of 800 UI/Kg subcutaneously twice a week, according to described protocols¹⁰. The total weekly dosage was not adjusted for weight gain, resulting in a progressive tapering of the dosage. The treatment significantly ameliorated the degree of anemia allowing transfusion-independence (Table 1). A second bone marrow aspirate at 5 months of age showed hypercellular marrow, an increased proportion of immature erythroblasts with non-specific dysplastic changes. A few erythroblasts had poorly-hemoglobinized cytoplasm and rings of coarse basophilic granules. To exclude hereditary sideroblastic anemia Perls’ staining of the marrow smears was performed, but search of ring sideroblasts was negative.

During the following years, Hb level was constantly maintained between 7 and 9.5 g/dL. Attempts to discontinue rEpo treatment performed at 2 and 4 yrs of age failed, since Hb
immediately dropped. At the age of 5 years a non invasive measurement of liver iron was performed by SQUID (Superconductum Quantum Interference Device) biomagnetic susceptometry. This method quantifies the magnetic effect of the hemosiderin and ferritin iron complexes (paramagnetic properties) throughout a superconducting loop.\textsuperscript{11-12} The results are quantitatively equivalent to those obtained by chemical analysis of tissue biopsy\textsuperscript{12}. A remarkable increased Liver Iron Concentration (LIC) (2536 ± 78 µg Fe/g hepatic wet weight) was determined, equivalent to 14.2 mg/gr liver dry weight and to a total body iron of 150.5 mg/Kg body weight\textsuperscript{13}. A liver sample of 1.2 cm was obtained by needle biopsy. Six portal spaces were evaluable. Normal architecture with mild enlargement of portal spaces was observed, as well as minimal focal inflammatory infiltrates. At Perl staining a severe iron overload was detected (Grade III according to Sciot\textsuperscript{14}), involving both hepatocytes and Kupffer cells. The iron distribution was predominantly on zone 1 (Figure 1) . Family history was unremarkable. Both parents had normal iron parameters. I-2 had borderline low MCV values. Relevant clinical and laboratory data of the proband and parents are reported in Table 1.

Methods

After informed consent, blood was obtained for genetic analysis from the proband and his parents. Blood from healthy control subjects was obtained after informed consent provided according to the Declaration of Helsinki. Approval was obtained from the Federico II University Medical School institutional review board for these studies.

Nucleotide sequence analysis of \textit{TFR1}, \textit{GATA1} and \textit{DMT1} from genomic DNA

Anticoagulated (EDTA-treated) blood samples were obtained and stored at -20°C. DNA samples were extracted from 0.2 ml of whole blood according to standard procedure\textsuperscript{15}. To screen for mutations of \textit{transferrin receptor 1 gene (TFR1)} for the patient and his parents each of the 18 exons with their intron/exon boundaries was amplified by polymerase chain reaction (PCR) using specific primers. PCR fragments were sequenced directly (see below). A similar approach was used to analyse \textit{GATA-1}: all coding exons including splice junctions, and portion of the promoter region were amplified by PCR and amplified fragments were directly sequenced. The \textit{GATA-1} and \textit{TFR1} cDNA sequences from GenBank accession number NM_002049 and M_003234 respectively were used as reference sequences. To analyse \textit{DMT1} all seventeen exons and the flanking intronic sequences were amplified by PCR using specific primers. Detailed protocols and primers sequences are available on request.
The amplified products were isolated by electrophoresis on 1% agarose gel and purified using the QIAamp purification kit (Qiagen). Direct sequencing was performed using a fluorescent-tagged dideoxy chain terminator method in an ABI 310 automated sequencer (Applied Biosystem, Foster City, CA USA), according to the manufacturer’s instructions. The DMT1 cDNA sequence from GenBank accession number NM_000617 was used as a reference sequence, where the A of the ATG translation initiation start site represents nucleotide +1.

DNA samples from 50 healthy individuals (100 chromosomes) of Caucasian origin were investigated for the identified DMT1 mutations, using suitable restriction enzymes. Digestion of the appropriate PCR products by endonucleases MboII and Hinf I (New England Biolabs, Beverly, MA) was according to manufacturer instructions. Cleavage products were evaluated on 2% agarose gels.

Reverse transcription–PCR amplification of DMT1

Total RNA was prepared from whole blood of healthy controls, the patient and his parents using blood RNA extraction kit (PreAnalytix, Qiagen) and following manufacturer's instructions. Total RNA was reverse transcribed in a 20 µL reaction using Superscript III reverse transcriptase (Invitrogen, Life Technologies, Inc., Carlsbad, CA) and specific DMT1 reverse primer 5’- CCTAAGCCTGATAGAGCTAG-3’. Amplification of DMT1 cDNA was performed using 3 sets of primers encompassing specific coding exons. Segment 1 (exons 4-6) forward primer: 5’- CACCGGACCAGGTTTTCTTA-3’, reverse primer: 5’- GATAGCAATGGCTGAGCC -3'; Segment 2 (exons 12-14) forward primer: 5’- TCATGGAGGGATTCCTGAAC -3', reverse primer: 5’-ATGAGAGCAAAGGGAAGCTG -3'. Segment 3 (exons 9-13) was studied in order to evaluate exon 12 skipping: forward primer: 5’- CAGGTACTCAAGGGCATGT -3', reverse primer: 5’-GTTGAGGAATCCCTCCATGA -3'. Two µL cDNA were used in each PCR reaction, 1x PCR buffer, 2 mM dNTPs, 2 U to 5 U Taq polymerase in a 50 µL reaction mixture. After initial denaturation at 95°C for 5 minutes, amplification was performed for 30 cycles for 95°C for 30 seconds, 60°C for 30 seconds, and 72°C for 30 seconds, then 72°C for 10 minutes. PCR products were analyzed on 1% agarose gels then purified on a QIAquick PCR purification column (Qiagen, Valencia, CA). Sequencing was performed as described above.

PBMC total protein lysate.

Isolation of peripheral blood mononuclear cells (PBMC) from patient, his parents and an healthy individual was performed by density gradient centrifugation from heparinized blood.
Total proteins were extracted by using a lysis buffer containing 10 mM Tris-HCl buffer (pH 7.5) and protease inhibitors (PIs; 2 µg/mL leupeptin; 2 µg/mL aprotinin, 1 µg/mL pepstatin; 100 µg/mL phenylmethylsulfonyl fluoride [PMSF] and 2 mM EDTA). All procedures were carried out at 4°C. Samples were stored frozen at -80°C. Protein concentration was determined by the Bradford assay (Bio-Rad).

**Immunoblotting**

For DMT1 detection, 80 µg of total protein lysate were separated by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE; 10% polyacrylamide), and transferred by electroblotting to polyvinylidene fluoride membranes. Because heat treatment of DMT1-containing samples was found to cause aggregation of the protein, samples were incubated for 30 minutes at room temperature in Laemmli buffer (with occasional vortexing) prior to SDS-PAGE. Loading and transfer of proteins were verified by staining the blots with Ponceau S (Sigma). Immunoblots were preincubated with blocking solution (0.02% Tween 20, 7% skim milk in PBS) for 3 hours at 20°C, prior to incubation with primary antibodies for 16 hours at 4°C in blocking solution. For immunoblotting, antibodies were used as follows: rabbit anti-DMT1 NT (1/200; a generous gift of Dr. Philippe Gros, McGill University, Montreal, QC, Canada), rabbit anti–β-actin (1/2000). After incubation with primary antibody, the blots were washed with TTBS, then incubated with alkaline phosphatase–labeled anti-rabbit secondary antibody (1/2000) at room temperature for 90 minutes. Immunodetection was obtained using enhanced chemiluminescence reagents (Cell Signaling Technology, New England Biolabs). Densitometric analysis was performed using Quantitative One 4.5.0. program (Bio Rad, Hercules, CA).
Results

We identified a male child from non consanguineous parents, who came to medical attention at birth because of severe hypochromic microcytic anemia. Beta thalassemia was not consistent with the presentation at birth and with the hemoglobin electrophoretic pattern of both parents. Globin chain synthesis suggested a mild alpha-thalassemia phenotype. However, the hypothesis of an alpha-thalassemia condition as the cause of the severe anemia was ruled out because no Hb H and Hb Bart’s were found at Hb electrophoresis (data not shown). In addition, analysis of the α-globin genes by means of southern blot and sequencing of the entire alpha cluster was normal. Sideroblastic anemia was excluded by bone marrow examination. Because of the severe poikilocytosis we considered also the possibility of hereditary pyropoikilocytosis (HPP), but peripheral blood smears of the parents showed no evidence of elliptocytosis and spectrin dimer percentage was normal (data not shown).

The proband hematological data suggested a condition related to an abnormality of iron metabolism, mimicking iron deficiency (hypochromia and FEP increase). However, serum iron, transferrin saturation and serum ferritin were increased before treatment with rEpo. No mutations were found in the HFE gene. Altogether the results suggested a defect of iron utilization.

Genomic analysis

In search for rare causes of microcytosis we sequenced TFRI and GATA genes without finding mutations in both genes. Then DMT1 coding sequence and exon-intron junctions were screened for mutations by direct sequencing.

Two novel mutations were identified in the proband both at the heterozygous state. The first was a 3 bp deletion in intron 4, c.310 - 3_5del CTT, that resulted in a splicing abnormality (Figure 2A). The second was a C>T transition at nucleotide 1246 in exon 13 (Figure 2B).

The 3 nucleotide deletion alters the acceptor site of intron 4, affecting the splicing process. This mutation was inherited by I-1. Mutant c.310 - 3_5del CTT abolishes a cleavage site for MboII endonuclease. We exploited this approach to investigate the sequence at this position in normal controls. The PCR products corresponding to intron4-intron 5 of genomic DNA of family members and normal controls were digested with MboII endonuclease. Wild-type PCR fragments (243bp) generated 2 smaller fragments of 63 and 180 bp that were present in all 100 control chromosomes, excluding the presence of the 3 bp deletion.
c.1246C>T changes the CGC codon to TGC, causing the substitution of arginine 416 with cysteine (p. R416C) in the protein. This mutation was inherited from I-2. c.1246C>T mutation also abolishes a *Hinf I* cleavage site. Restriction analysis performed using *Hinf I* endonuclease on amplified exon 13 from family members and 50 controls revealed the absence of the site in I-2 and the proband at the heterozygous state and the presence of the normal *Hinf I* site in all control chromosomes (not shown). In addition using PROGRAMM blastn-SNP (http://www-btls.jst.go.jp/cgi-bin/Homology_Blast-SNP/submission_v3.cgi?PROGRAM=blastn-SNP) we excluded that this nucleotide change corresponds to a previously identified SNP.

The p. R416C mutation affects with a non conservative substitution a residue that is highly conserved across species and also in the DMT1 (or Nramp2) homologous protein Nramp1 (Figure 2C). The mutation is localized in one of the putative DMT1 transmembrane domains (VVLTRSIAILPTLLVA) as identified by PSORT II analysis, (K. Nakai http://psort.nibb.ac.jp).

**Analysis of DMT1 mRNA.**

In order to determine the effect of both mutations *DMT1* cDNA of patient and parents blood cells was analysed. Specific cDNA fragments that span regions encompassing the mutations (exons 4-6 and 12-14 respectively) were amplified by PCR and the fragments directly sequenced. Amplification of exon 4-6 region of *DMT1* from normal whole blood mRNA produced a single transcript of the expected size (298 bp). By contrast two transcripts were obtained from patient and I-1 cDNA, one corresponding to the expected size segment and a shorter one of approximately 180 bp (Figure 3A). Quantification of the two bands indicates that the smaller band lacking exon 5 was approximately 30-35 % of the total in both cases. Nucleotide sequencing showed that the 298 bp band contains the normal, full-size message, whereas the smaller band corresponds to a transcript with deleted exon 5. The improperly spliced RNA lacking exon 5 retains the correct reading frame, but the encoded protein lacks 40 amino acids (Figure 3B). The deleted region includes a putative transmembrane domain, FKLLWILLLATLVLGLL, predicting a defect in the correct insertion of the protein into the plasma membrane or a defective iron transport.

Sequencing of the amplified fragments corresponding to exons 12-14 confirmed the presence of the heterozygous c.1246C>T mutation in exon 13 both in the proband and in I-2.

Since exon 12 skipping was previously observed in minimal amounts in hematopoietic cells of healthy subjects and was reported to be exaggerated in a patient with *DMT1* homozygous mutation⁹ we studied exon 12 skipping in all family members. Studying exon 9-13 regions of
DMT1 mRNA we demonstrated the presence of two transcripts, whose size corresponded to variants with or without exon 12 respectively in all family members. The smaller product was present in low proportion (less than 10%), as described for normal controls.

**Protein expression**

To evaluate the effect of the DMT1 mutations identified, expression of DMT1 protein was then investigated in the PBMC of the patient, his parents and a healthy control. Immunoblots were analyzed using the anti-DMT1-NT antibody. A single band of approximately 65-kDa was present in all samples and its expression was decreased in the proband (about 40% of the total) and in I-1. On the contrary, DMT1 protein in I-2 was similar to that observed in controls. These findings confirm that the paternal allele causes a quantitative defect of DMT1 protein, whereas the maternal allele with a missense mutation has no effect on the protein amount.

**Discussion**

Here we report the case of a child affected by compound heterozygosity for two different DMT1 mutations. He had severe microcytic-hypochromic anemia from birth. The diagnosis was reached after common causes of microcytosis were excluded. Since in animal models heterozygosity for TFR1 deletion may cause iron deficiency, we sequenced patient TFR1 gene, without finding mutations of the coding regions or exon/intron junctions. We sequenced also GATA 1, since mutations of this gene were reported to cause microcytic anemia in males, but causal mutations were not detected. The alpha-thalassemia-like picture and the high levels of FEP might have been induced by iron deficiency. However, high serum iron, transferrin saturation and serum ferritin addressed to a disorder of iron utilization.

Sequence analysis of DMT1 of the proband revealed a compound heterozygosity for two novel mutations, one affecting the process of RNA maturation and the second introducing a non-conservative amino acid substitution in a putative transmembrane domain. The effect of the mutation in the consensus sequence of the acceptor splice site of intron 4 on the splicing process was demonstrated by the finding of consistent amounts of abnormal transcripts with deleted exon 5 in DMT1 cDNA from blood cells of II-1 and I-1 (Figure 3).

Skipping of exon 10 and 12 may occur during DMT1 mRNA maturation. However, skipping of exon 5 as a physiological process was never reported previously. The predicted protein lacking 40 amino acids of the first putative transmembrane domain might be destabilized, non-functional and/or degraded. A protein reduction is confirmed by immunoblotting studies in both carriers of the mutation (I-1 and II-1, Figure 4). As shown by the normal hematological and
iron parameters of I-1 the mutation is well tolerated at the heterozygous state, likely because a sufficient DMT1 amount is provided by the normal trans allele.

The maternal mutation (p. R416C) predicts a non-conservative amino acid change in exon 13. In the absence of functional data, it is difficult to assess whether p. R416C represents a causal mutation. Normal control studies and database search ruled out a common polymorphic change. It is likely that residue 416 is relevant for the protein function, since it is highly conserved across species and localized in a putative (the 7th) transmembrane domain. Also the G185R mutation identified in mk mouse and b rat occurs in a predicted transmembrane domain (the 4th). Recently, in cell lines stably transfected with G185R DMT1 it was shown that only a proportion of the abnormal protein reaches the membrane and the endosomal compartment, whereas a large fraction is retained in the endoplasmic reticulum and rapidly degraded by a proteasome-dependent mechanism. Low levels of exon 12 skipping were reported physiologically in blood cells and exaggerated skipping of exon 12 was interpreted as causal in E399D DMT1 mutation, reducing full-size RNA and producing a shorter protein lacking transmembrane domain, and unable to uptake iron. In our study dealing with different mutations, a transcript lacking exon 12 was present in minimal amount in all family members, indicating that missense p.R416C mutation does not interfere with the correct RNA processing. In addition ESE analysis excluded an enhancer role for the sequence encompassing R416C mutation. Also, the amount of the protein of I-2, heterozygous carrier of p. R416C mutation, was not reduced in PBMC western blot. I-2 has slightly decreased MCV, but no anemia neither abnormalities of iron parameters. The severity of the phenotype of the proband at birth is reminiscent of that observed in animal models, consistent with a fundamental role for DMT1 in erythroid cell production during fetal life. A mouse with complete inactivation of DMT1 (DMT1/-) was recently reported, whose phenotype at birth was more severe than that of mk mouse. The analysis of mice with selective DMT1 inactivation in the gut, bone marrow cells and liver reinforced its essential role for intestinal absorption and erythroid utilization, but not for liver iron uptake, since hepatocyte iron storage was only incompletely interrupted. The history of II-1 strengthens the DMT1 role in erythroid cells, but clearly indicates that DMT1 is not essential for liver iron uptake also in humans.

Iron stores were elevated early in life, concomitant with severe anemia, as shown by ferritin levels. During follow up a decreased ferritin was recorded following rEpo treatment and Hb increase, likely reflecting iron utilization. Intriguingly, levels of serum ferritin were disproportionately low faced with the severe iron burden, demonstrated both at SQUID and at
liver biopsy. The high liver iron content relative to the age and the estimated high total body iron\textsuperscript{13}, was disproportional to the iron received from transfusion or redistributed from the marrow, but requires a significant increase of intestinal absorption. We have not measured DMT1 protein in the gut, and in principle sufficient functional DMT1 could be not rate-limiting for basal iron absorption at this level. However, increased iron absorption might occur through DMT1-independent pathways, possibly involving upregulation of an iron-heme pathway of absorption\textsuperscript{8}.

The proband severe anemia required blood transfusions until rEpo treatment allowed to reach an hemoglobin level compatible with growth and development, as demonstrated by the weight and height growth curves (Table 1 and data not shown). A spontaneous improvement in the degree of anemia is unlikely, as the attempts to reduce rEpo administration resulted in rapid worsening of anemia. The rEpo response of our patient is in agreement with what observed in vitro by Priwizerova et al\textsuperscript{8}, since the number and size of the erythroid colonies of their patient were significantly increased when grown in the presence of the Fe-SH iron donor chelate and/or high Epo levels\textsuperscript{22}. Epo might have a non specific effect on erythropoiesis, reducing apoptosis of the erythroid precursors, or a specific one, inducing a DMT1-independent pathway of iron utilization. Based on the observation that loss of \textit{TFRI} is more severe than loss of \textit{DMT1}, transporters other than DMT1 in the endosomal cycle have been hypothesized in animals\textsuperscript{21}. Since no variations were observed in MCV and MCH of the proband, following partial anemia correction by Epo (Table 1), we favour the interpretation that Epo ameliorates total erythropoiesis. Because of the high iron content at liver biopsy and SQUID, iron chelation by deferioxamine was prescribed. The simultaneous administration of Epo and deferoxamine, apparently paradoxical, is justified by the dual face disorder with features of both anemia and iron overload. However, this treatment will require careful monitoring of iron parameters and periodic non invasive assessment of liver iron.

When DMT1 was initially identified as the gene encoding an intestinal iron transporter it was predicted that a subset of human patients with congenital anemias might harbor mutations in this gene\textsuperscript{4}. The possibility of \textit{DMT1} defects, although rare, should be considered in case of microcytosis, especially at birth, once thalassemia and HPP are ruled out, or after exclusion of sideroblastic anemia, in patients with atypical microcytic anemia and elevated iron parameters.
Legend to Figures

**Figure 1**: Micrographs of liver biopsy showing the entity and the distribution of iron. Staining technique: Perl Prussian blue. (A) The liver has normal architecture. The iron is heavily distributed in zone 1, whereas zone 2 and 3 appear less loaded. Original magnification x 25. (B) Most hepatocytes are massively loaded by hemosiderin (Sciot grading 3), as well as Kupffer cells to a minor extent (Sciot grading 2). Original magnification x 200.

**Figure 2**: Identification of the DMT1 mutations in the proband.
(A) Partial sequence of intron 4-exon 5 junction of the proband and wildtype (WT) DNA identifying the c.310 - 3_5del CTT mutation (underlined). The abnormality in the consensus sequence of the acceptor splice site is shown on the right. The dinucleotide acceptor site is in bold. (B) Sequence analysis of the DNA region encompassing exon 13 showing the heterozygous C>T transition at position 1246 causing p. R416C mutation. (C) ClustalW alignment of the amino acid sequences of NRAMP2(DMT1) and NRAMP1 orthologs from different species, showing complete conservativeness of the arginine 416 R residue (boxed).

**Figure 3**: Analysis of the effect of c.310 - 3_5del CTT deletion.
(A) Amplification of exon 4-6 region of DMT1 mRNA from whole blood. cDNA was synthesized as described in Methods. The exon 4-6 region of DMT1 cDNA was amplified by PCR and the product separated on 1% agarose gel. Lane 1. 1Kb DNA ladder; Lane 2. I-1; Lane 3. Proband; Lane 4. I-2; Lane 5. normal control; Lane 6. no template control. The upper band is 298 bp, the lower band in lanes 2 and 3 is 178 bp. (B) Sequencing of PCR product of the 178 bp band (A) of II-1, resulting in skipping of exon 5 and 40 amino acid loss in the protein. (C) Schematic representation of DMT1 exon 5 skipping in the proband.

**Figure 4**: Detection of DMT1 protein in PBMC
Total PBMC extracts were prepared as described: 80 µg total proteins were loaded into each lane and separated by SDS-PAGE on a 10% acrylamide gel followed by transfer to PVDF membranes. Membranes were incubated with anti-DMT1-NT antibody (A), and with β-actin antibody as an internal loading control (B). Samples in the different lanes are indicated. Sizes in kDa are on the right.
Acknowledgments:
We are indebted to Philippe Gros for the generous gift of the anti-DMT1 antibody.
We thank Renzo Galanello for performing Southern Blot and sequencing of the alpha globin genes and Mario Cazzola for Perls reaction on bone marrow of the proband. Furthermore we thank Arturo Romondia for its clinical advice.
Table 1. Relevant clinical and laboratory data of the proband (at different ages) and parents

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<th>Mother (I-2)</th>
<th>Proband (II-1)</th>
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M = months; Bw = body weight; TS = Transferrin saturation; FEP = free erythrocyte protoporphyrin; Epo = rErythropoietin, starting dose: 800 UI/Kg s.c. x 2/ weekly (see text for details)

PRBC= packed red blood cells
References
alternative to blood transfusions in infants with hereditary spherocytosis - The Hematology Journal 2000; 1: 146-152.


DMT1 NT antibody

β-actin antibody

Ctrl  I-2  II-1  I-1

75

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Microcytic anemia and hepatic iron overload in a child with compound heterozygous mutations in DMT1

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