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Missense *SLC25A38* variations play an important role in autosomal recessive inherited sideroblastic anemia

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ABSTRACT

Background

Congenital sideroblastic anemias are rare disorders with several genetic causes; they are characterized by erythroblast mitochondrial iron overload, differ greatly in severity and some occur within a syndrome. The most common cause of non-syndromic, microcytic sideroblastic anemia is a defect in the X-linked 5-aminolevulinate synthase 2 gene but this is not always present. Recently, variations in the gene for the mitochondrial carrier *SLC25A38* were reported to cause a non-syndromic, severe type of autosomal-recessive sideroblastic anemia. Further evaluation of the importance of this gene was required to estimate the proportion of patients affected and to gain further insight into the range and types of variations involved.

Design and Methods

In three European diagnostic laboratories sequence analysis of *SLC25A38* was performed on DNA from patients affected by congenital sideroblastic anemia of a non-syndromic nature not caused by variations in the 5-aminolevulinate synthase 2 gene.

Results

Eleven patients whose ancestral origins spread across several continents were homozygous or compound heterozygous for ten different *SLC25A38* variations causing premature termination of translation (p.Arg117X, p.Tyr109LeufsX43), predicted splicing alteration (c.625G>C; p.Asp209His) or missense substitution (p.Gln56Lys, p.Arg134Cys, p.Ile147Asn, p.Arg187Gln, p.Pro190Arg, p.Gly228Val, p.Arg278Gly). Only three of these variations have been described previously (p.Arg117X, p.Tyr109LeufsX43 and p.Asp209His). All new variants reported here are missense and affect conserved amino acids. Structure modeling suggests that these variants may influence different aspects of transport as described for mutations in other mitochondrial carrier disorders.

Conclusions

Mutations in the *SLC25A38* gene cause severe, non-syndromic, microcytic/hypochromic sideroblastic anemia in many populations. Missense mutations are shown to be of importance as are mutations that affect protein production. Further investigation of these mutations should shed light on structure-function relationships in this protein.

Key words: congenital sideroblastic anemia, *SLC25A38*, missense mutations.

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The online version of this article has a Supplementary Appendix.

Introduction

Congenital sideroblastic anemias are heterogeneous, rare disorders characterized by aregenerative anemia of varying severity, hypochromic peripheral erythrocytes and decreased heme synthesis. In the bone marrow an increased percentage of ringed sideroblasts formed by iron-loaded mitochondria clustered around the erythroblast nucleus can be visualized by Perls' staining. Systemic iron overload secondary to chronic ineffective erythropoiesis is an important feature of most congenital sideroblastic anemias. Different forms of these anemias are defined at the molecular level, each of which has provided insight into cellular pathways associated with dysfunctional mitochondrial iron metabolism. Some congenital sideroblastic anemias are syndromic while in others microcytic anemia and subsequent iron overload are the sole manifestations.

X-linked sideroblastic anemia (XLSA, MIM# 300751) is the most common non-syndromic genetic form and results from mutations in the erythroid-specific gene encoding 5-aminolevulinate synthase 2 (ALAS2, EC 2.3.1.37). ALAS2 is the initial enzyme of the pathway of heme biosynthesis in erythroid cells and catalyzes the condensation of glycine and succinyl-coenzyme A into 5-aminolevulinic acid, using pyridoxal phosphate as an essential cofactor. The onset of symptoms in patients with X-linked sideroblastic anemia ranges from birth to the eighth decade; the anemia is generally mild to moderate and often responds to pyridoxine.¹

Two recent reports indicate that variations in the newly-described *SLC25A38* gene can be responsible for severe pyridoxine-refractory congenital sideroblastic anemia. This anemia was markedly microcytic and inherited in an autosomal recessive manner.^{2,3} The patients could present in the first few months of life and were often dependent on regular blood transfusions for normal development. Bone marrow transplantation was, therefore, considered as a cure and has been successful in two of four published cases.

SLC25A38 is located on chromosome 3p22.1 and encodes a mitochondrial carrier protein required for erythropoiesis. This protein is thought to be located in the inner mitochondrial membrane and may act by importing glycine into mitochondria or by exchanging glycine for 5-aminolevulinic acid. Most of the variations reported affect protein levels (nonsense, frameshift or splice site variations) but three missense variations have also been found (p.Gly130Glu, p.Arg134His, p.Arg187Pro).^{2,3} To gain further insight into the nature of this type of disorder and the prevalence and nature of *SLC25A38* variations we report here the genetic diagnosis performed on patients with congenital sideroblastic anemia negative for *ALAS2* mutations.

Design and Methods

Three laboratories or clinical centers offering diagnostic services for congenital sideroblastic anemia analyzed the *SLC25A38* genomic sequence in 24 patients. Eleven patients were studied in Cardiff, 12 in Paris and one in Barcelona. None of these patients was included in the studies already published.^{2,3} The criteria for inclusion differed slightly for the three laboratories; however, shared minimal criteria were non-syndromic congenital sideroblastic

anemia (i.e. ring sideroblasts in the bone marrow with no known secondary cause, an absence of features linked to known syndromic types of congenital sideroblastic anemia and an absence of indication in favor of a diagnosis of myelodysplastic syndrome), anemia refractory to pyridoxine and folic acid and an absence of the *ALAS2* mutation.

The conditions of DNA extraction, polymerase chain reaction and sequence analysis used in the three laboratories were standard and are reported in the *Online Supplementary Appendix*. All exons, exon-intron boundaries and a varying amount of the 5' and 3' flanking sequence of the *SLC25A38* gene were examined using fluorescent chain-terminator cycle sequencing. Newly designed or previously published² primers were employed (*Online Supplementary Table S1*).

All participants or their parents, as appropriate, had given informed consent for genetic diagnosis, in keeping with the respective regulations from the countries in which the diagnosis was performed. In one case, the diagnosis was performed as part of a broader research study (HMA-IRON ERARE-155) approved by the local research ethics committee.

Blood samples from both parents were available, except for patients 9 and 11 (Table 1). Samples from four additional members of three families were also studied.

Multiple sequence alignment of *SLC25A38* proteins from 14 different species was performed using Clustalw2 (<http://www.ebi.ac.uk/Tools/clustalw2/>).⁴ Mitochondrial carrier protein domains (PF00153) are shown as defined by Pfam (<http://pfam.sanger.ac.uk/>) on sequence S2538 HUMAN.⁵ Potential transmembrane helical regions of human *SLC25A38* were obtained from Uniprot (<http://www.uniprot.org>) and from the secondary structure prediction of the 3D protein modeling. The protein 3D-modeling was achieved with Swiss-model web-server (<http://swissmodel.expasy.org/>) in automated mode using the PDB structure of carboxyatractyloside-inhibited bovine mitochondrial ADP/ATP carrier (2c3e) as a template.^{6,7} The structures were superimposed and the mutations visualized using the open-source software PyMOL™ 0.99rc6 (<http://www.pymol.org/>).⁸

Results

We studied 24 probands with manifest symptoms of congenital sideroblastic anemia, ring sideroblasts in the bone marrow, anemia refractory to treatment with pyridoxine and folic acid, and no *ALAS2* mutations. Of these, 11 were found to have inherited ten different *SLC25A38* variations that are likely causative of their congenital sideroblastic anemia. These 11 patients, with 15-73% ring sideroblasts in the bone marrow erythroblasts, were of several different ancestral origins (4 Spanish, 1 Portuguese, 1 Algerian, 2 Moroccan, 1 Egyptian, 1 French and 1 Sri Lankan); three were male and eight were female. Additional relevant features of the patients and details of the mutations are presented in Table 1. The anemia was of early onset although not necessarily present at birth. All patients required blood transfusions, sometimes occasional but inevitably becoming regular, usually in the first few years of life. This dependence appears delayed in two patients (numbers 8 and 9, Table 1), one of whom did not receive regular blood transfusions until he was 20 years old (patient number 9, Table 1). Increased transferrin saturation was observed in five patients (patients 4, 6, 7, 10 and 11), accompanied in three by increased serum ferritin (patients 6, 7 and 10), before significant amounts of blood had been received.

Iron overload remains a problem in two patients (patients 4 and 6) despite iron chelation.

Nine patients were homozygous and two (patients 4 and 11) were compound heterozygotes for *SLC25A38* variations found in five (exons 2 and 4-7) of the seven exons (Online Supplementary Figure S1). In all instances studied, the parents were found to be heterozygous for the same variation as found in the respective proband. Unaffected siblings or relatives tested were found to have inherited only the usual alleles or were heterozygous for the variation found. None of these variations was identified as a polymorphism in the Ensembl (www.ensembl.org/Homo_sapiens/Info/Index) or NCBI (www.ncbi.nlm.nih.gov/) single nucleotide polymorphism databases last searched 14.02.2011; the p.Gly228Val variant has not been detected in 90 North European control alleles studied.

Two mutations (p.Arg117X and p.Gly228Val) were found in the homozygous state in two unrelated families. All but two variations described here are missense mutations while one is a nonsense variation (p.Arg117X) and one is a deletion leading to a frame shift mutation p.Tyr109LeufsX43 (Figure 1). The involvement of

p.Arg117X, p.Tyr109LeufsX43 and of p.Asp509His variations in congenital sideroblastic anemias has been described previously² but seven variations are reported here for the first time. Two new mutations (p.Arg134Cys and p.Arg187Gln) involve amino acids already known to be affected by a different substitution (p.Arg134His and p.Arg187Pro) in patients with congenital sideroblastic anemias.² The previously described missense variation p.Asp209His, formed by a G to C transversion at the end of exon 5, is predicted to abolish a splice donor site,² thereby leading to a truncated protein and possibly rapidly degraded mRNA by nonsense-mediated mRNA decay. All seven new mutations result in substitutions of conserved amino acids (Figure 1) situated in predicted transmembrane regions forming the three mitochondrial carrier domains (Figures 1 and 2A). Structure analysis shows that five of the seven substitutions affect amino acids with side chains oriented toward or extending into the central transport channel, consistent with a plausible mechanistic role of the newly described mutations in transport activity (Figure 2B; additional detail can be found in Online Supplementary Figure S2).

Table 1. Mutations of the *SLC25A38* gene and main features of the patients.

Patient number	Age of presentation	Hemoglobin at diagnosis, g/dL (age)	Mean corpuscular volume fl. at diagnosis	Treatment	Transferrin saturation (age)	Ferritin µg/L (age)	Lowest hemoglobin during treatment g/dL (age)	Mean corpuscular volume fl. at lowest hemoglobin	¹ HGVS names of mutations found in <i>SLC25A38</i> gene ENSG00000144659; Genbank mRNA: NM_017875; Genbank protein: NP_060345
1	5 months	6.2	61.6	Blood transfusion, occasional then regular	not available	not available	2.2	not available	c.[569 C>G]+[569C>G] p.[Pro190Arg]+[Pro190Arg]
2	4 months	7.2	62	Blood transfusion, bone marrow transplant	not available	371 (<1 year)	6.2	60.4	c.[683G>T]+[683G>T] p.[Gly228Val]+[Gly228Val]
3	2 months	7.2	76	Blood transfusion, regular	not available	577 (<1 year)	3.9	76	c.[683G>T]+[683G>T] p.[Gly228Val]+[Gly228Val]
4	birth	6.9	61.3	Blood transfusion, regular	84% (7 months) 81% (3 years)	136 (7 months) 1340 (3 years)	6.7 (18 months)	73	c.[560G>A]+[625G>C] p.[Arg187Gln]+[Asp209His]
5	birth	6.5 (at birth)	65.3	Blood transfusion, occasional then regular; bone marrow transplant	100% (2-3 years)	548 (2-3 years)	5.8 (20 months)	74	c.[349C>T]+[349C>T] p.[Arg117X]+[Arg117X]
6	2 days	10.3	71	Blood transfusion from 2 years	71% (2 days) 71% (15 years)	398 (2 days) 1492 (15 years)	5.9 (2 years)	65	c.[440T>A]+[440T>A] p.[Ile147Asn]+[Ile147Asn]
7	15 days	9	55	Blood transfusion from 2 years	81% (2 years)	740 (2 years)	5.5 (1 year)	62	c.[166C>A]+[166C>A] p.[Gln56Lys]+[Gln56Lys]
8	3 years	7	50	Blood transfusion, occasional	95% (12 years)	537 (12 years)	5.5 (12 years)	48.5	c.[832C>G]+[832C>G] p.[Arg278Gly]+[Arg278Gly]
9	2 years	not available	not available	Blood transfusion, occasional then regular	72% (22 years)	1000 (22 years)	4.5 (21 years)	55	c.[400C>T]+[400C>T] p.[Arg134Cys]+[Arg134Cys]
10	1 month	5.1	80	Blood transfusion, regular, bone marrow transplant	56.2% (1 month) 91% (3 years)	618 (1 month) 997 (3 years)	4.1 (3 years)	67	c.[349C>T]+[349C>T] p.[Arg117X]+[Arg117X]
11	14 months	6.5	55	Blood transfusion, regular from 14 months	99% (14 month)	not available	not available	not available	c.[324_325delCT]+[349C>T] p.[Tyr109LeufsX43]+[Arg117X]

¹HGVS: Human Genome Variation Society recommended mutation descriptions; c refers to the coding region nucleotide(s) affected with A of the initiation ATG codon as 1; p refers to the protein amino acid(s) affected with the initiation methionine as 1.

Discussion

Our observations confirm and significantly extend the findings in previous studies regarding *SLC25A38* variations in congenital sideroblastic anemias.^{2,3} In Paris, out of all 34 probands referred with congenital sideroblastic anemias (syndromic and non-syndromic), 15 (44%) had been found to have *ALAS2* variations and, as reported here, *SLC25A38* variations were found in five (15%). These percentages are similar to those found by Bergmann *et al.*³ (37% *ALAS2*, 15% *SLC25A38*) in a cohort of 83 probands. In Cardiff the percentages were lower, probably because of different criteria for accepting referrals. Out of 71 probands with congenital sideroblastic anemias referred to Cardiff, 17 (24%) had *ALAS2* variations and *SLC25A38* variations have been found in five (7%). Despite the diagnosis of additional genetic causes³ in the Paris and Cardiff laboratories, and a significant number of cases in which *SLC25A38* was not investigated, the *SLC25A38*-associated congenital sideroblastic anemias currently represent the second most common genetically-defined type referred to our laboratories, as was previously reported.³

None of the heterozygous carriers was affected in any discernible way. In one family (patient 5) the mother and the proband were heterozygous for hemoglobin Lepore without any noticeable interaction between the two

unusual genotypes. All patients described here were severely anemic with markedly microcytic and hypochromic red blood cells. The severity and refractory nature of the anemia led to a dependence on regular blood transfusion from an early age, and for some to seek compatible bone marrow donors for transplantation. Two patients in this series who have already undergone this procedure (patients 2 and 5) have been successfully engrafted with a follow-up time of 3 years and 4 months, respectively. Patient 10 is currently undergoing this procedure with no significant toxicities so far. These successes strengthen the case for offering this procedure as a possible cure to patients severely affected by this type of congenital sideroblastic anemia who have matched donors.

As with all types of dyserythropoietic disorders, in several patients studied here, there was evidence of increased iron absorption prior to blood transfusion. It is thought that the cause of this iron overload is repression of hepcidin expression produced by an expanded but ineffective erythropoiesis.^{9,10} In addition, all patients became rapidly transfusion-dependent so that regular monitoring of iron levels and iron chelation therapy to prevent complications of secondary iron overload are essential.

The correct molecular diagnosis in these patients was of great importance providing reassurance that the sideroblastic anemia is non-syndromic and enabling accurate

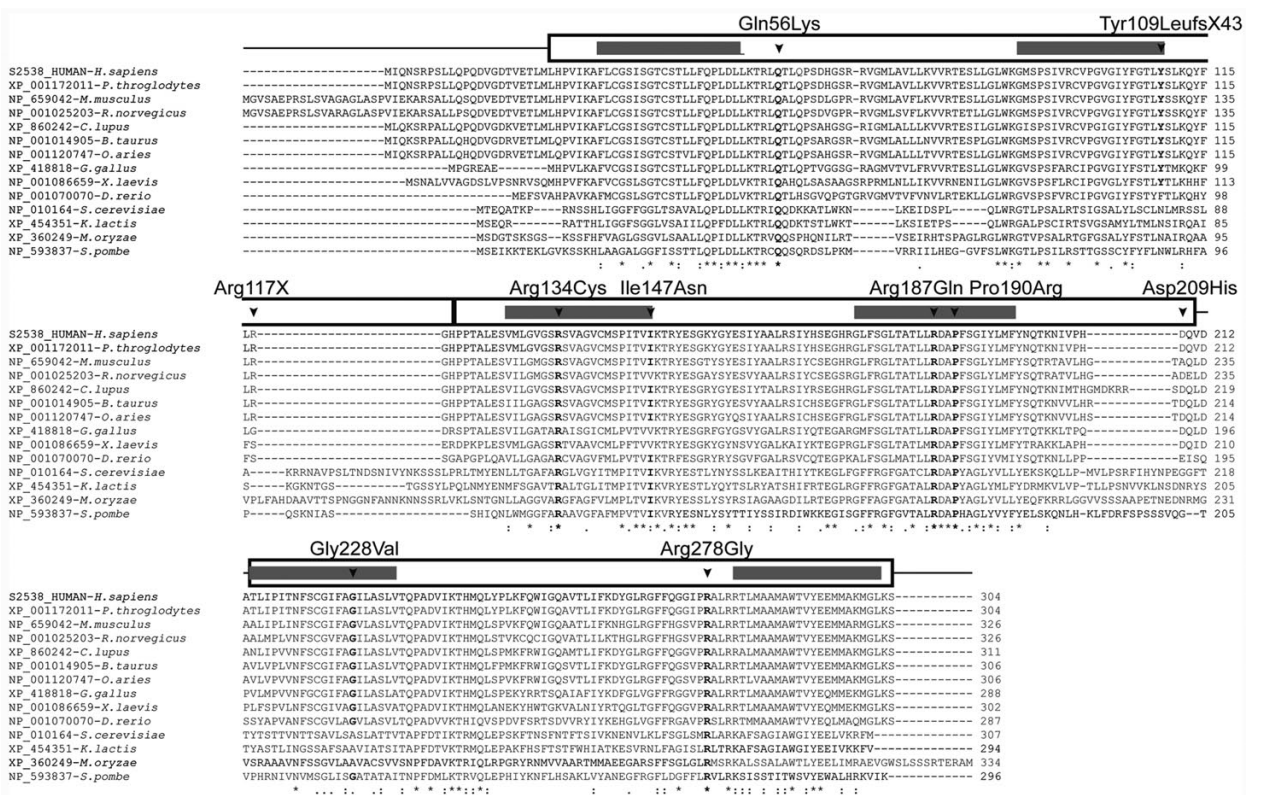


Figure 1. Multiple amino acid sequence alignment obtained using Clustalw2 for *SLC25A38* from 14 species (7 mammalian, 1 bird, 1 frog, 1 fish and 4 yeast/fungi). Amino acids reported in this work to be substituted in congenital sideroblastic anemia patients are marked with a vertical arrow. Shaded bars represent the transmembrane regions as reported by Uniport and are extended slightly by the structure modeling as shown in Figure 2. The mitochondrial carrier domains (Pfam) are shown as black boxes. Below the alignment a star indicates that the amino acid at this position is identical for all 14 species, dots indicate amino acids with similar but not identical properties.

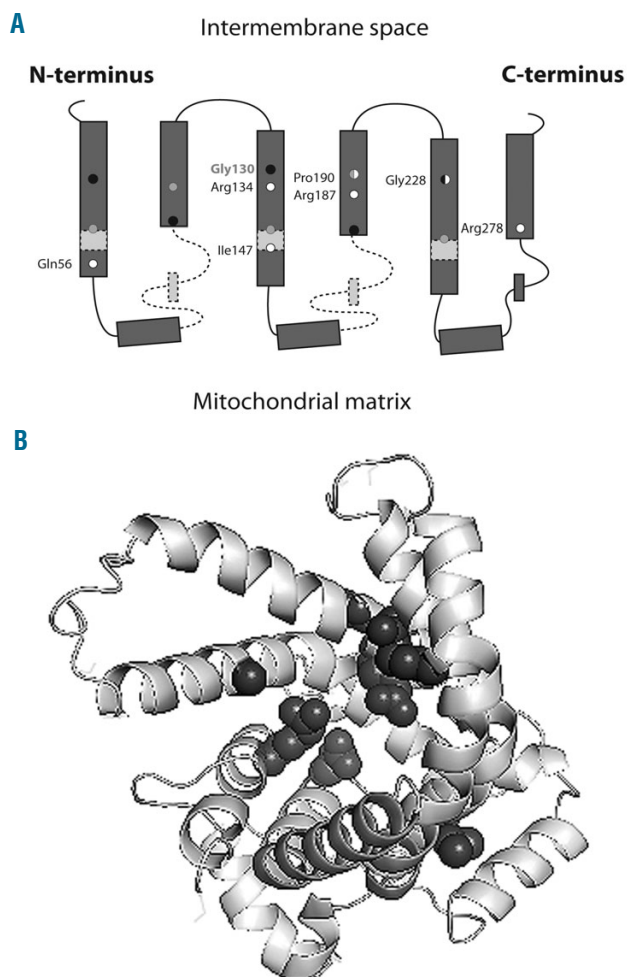


Figure 2. (A) The position of the substituted amino acids in the predicted secondary structure of human SLC25A38. Predicted helices are shown as blocks and predicted loops are shown as lines. The positions of the amino acid substitutions reported here are shown as white dots with respect to key glycine (black dot or half black-half white dot) and proline (gray dot or half gray-half white dot) residues. The latter were identified by their position in the amino acid sequence relative to the $PX[D/E]XX[K/R]X[K/R]$ and $[D/E]GX[XX[W/Y/F]][K/R]G$ parts of the signature sequence for this class of proteins (shown in a hashed line here). The position of the previously-reported substituted amino acid Gly130 (gray text) is also shown. (B) Predicted structural model by SWISS-MODEL of SLC25A38 visualized with PyMol. The backbone helices and loops are shown in light gray and the side chains of the substituted amino acids are shown as dark gray spheres. The side chains of five of the seven substituted amino acids can be seen to point into the central channel. The side chains of the 190 proline and the 147 isoleucine can be seen as the uppermost and the furthest right of the side chains respectively. The view is from the mitochondrial intermembrane space down the central channel but somewhat at an angle to show the 147 isoleucine side chain stretching away from the channel toward a short helical section that lies at the matrix side of the inner mitochondrial membrane. Please also refer to the *Online Supplementary Figure S2*.

genetic counseling on the risk of other cases within the family with the offer of prenatal diagnosis and preimplantation genetic diagnosis if required. It also allowed *SLC25A38* genotyping of potential bone marrow donors.

This study shows that more than half (55%) of the 18 implicated *SLC25A38* variations are missense as opposed to 27% prior to this report, which may provide further stimulus for the development of an *in vitro* drug discovery assay to correct the defect. It is also interesting to note that some patients appear to have a milder course of the disease (homozygous p.Arg134Cys and homozygous p.Arg278Gly in this report; homozygous p.Arg117X, compound heterozygotes p.[Arg134His]+[Tyr293X] and p.[X305ArgextX28]+[Asp209His] in a previous report²) for reasons apparently unrelated to the variations inherited, suggesting that additional environmental or genetic factors could play a role in disease penetrance.

The SLC25A38 protein is one member of a large family of mitochondrial carrier proteins.¹¹ Some of these carriers transport in only one direction, but most exchange one substrate for another. In the case of SLC25A38, the arginine-aspartic acid (RD) dipeptide at 187-188 putatively identifies it as an amino acid transporter. Yeast and zebrafish studies have suggested that SLC25A38 transports glycine into the mitochondrial matrix and exports aminolevulinic acid out of the mitochondrion to the cytosol. Although three-dimensional structure analysis has been reported for only one member of the mitochondrial carrier family, the bovine mitochondrial ATP/ADP carrier (PDB: 2c3e), protein homology modeling and mul-

tiples sequence alignment of all mitochondrial carriers indicate that they share certain important structural features.¹¹ These proteins consist of three tandem repeats of mitochondrial carrier domains. Each domain contains two transmembrane helices separated by a stretch of residues including the mitochondrial carrier signature motif encompassing a loop and an additional helical region (Figure 2A and *Online Supplementary Figure S2*).¹¹ The six transmembrane helices arrange themselves around a central cavity through which the solutes pass. Key amino acid residues, many of which are charged, have side chains that stretch into this cavity and play a critical role in the opening and closing of the channel gates or in the specific binding, transport and release of the substrates. In addition, certain conserved prolines and glycines are appropriately placed to allow the kinking and swiveling of the transmembrane helices required for transport to occur.^{7,11}

From structural similarity we hypothesize that the new reported missense mutations affect the transport function of SLC25A38 in different ways: mutations Gln56Lys and Arg278Gly will affect the gate to the matrix, mutations Arg134Cys and Arg187Gln will affect substrate binding or specificity and mutations Gly228Val, Pro190Arg and the already described Gly130Glu will affect the pivotal position for substrate binding and gate opening to the intermembrane space. The Ile147 does not appear to have a side chain that stretches into the channel but is a conserved hydrophobic residue that lies within a highly conserved motif at the matrix gate.

Overall, by similarity with mutations present in other mitochondrial carrier disorders, we believe the findings described here represent a step forward in the understanding of the nature of this type of congenital sideroblastic anemia.¹¹ Further investigation of these natural variants will enhance our knowledge of the function of this particular protein and its role in heme synthesis of developing red cells.

Online Supplementary Appendix

Further details of the methodology of DNA extraction and mutation analysis are provided in the *Online*

Supplementary Design and Methods. Online Supplementary Table S1 presents the primer sequences, while *Online Supplementary Figure S1* shows the sequence analysis results for each of the mutations found visualized by Mutation Surveyor software. *Online Supplementary Figure S2* is a color illustration showing the position of the substituted amino acids on the modeled three-dimensional structure.

Authorship and Disclosures

The information provided by the authors about contributions from persons listed as authors and in acknowledgments is available with the full text of this paper at www.haematologica.org.

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