Absent phenotypic expression of X-linked sideroblastic anemia in one of two brothers with a novel ALAS2 mutation

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Running title: Silent X-linked sideroblastic anemia

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Abstract

X-linked sideroblastic anemia (XLSA) is caused by mutations in the erythroid-specific 5-aminolevulinic acid synthase (ALAS2) gene. Hemizygous males have microcytic anemia and iron overload. A 38-year-old male presented with this phenotype (Hb 7.6 g/dL, MCV 64 fL, serum ferritin 859 µg/L) and molecular analysis of ALAS2 showed a mutation 1731G→A predicting an R560H amino acid change. A 36-year-old brother was hemizygous for this mutation and expressed the mutated ALAS2 mRNA in his reticulocytes, but showed almost no phenotypic expression. All five heterozygous females from this family, including the three daughters of the non-anemic hemizygous male, showed marginally increased red-cell distribution width (RDW). Although variable penetrance for XLSA in males has been previously described, this is the first report showing that phenotypic expression can be absent in hemizygous males. This observation is relevant to genetic counseling, emphasizing the importance of gene-based diagnosis.
Introduction

X-linked sideroblastic anemia (XLSA) is caused by mutations (primarily missense) in the erythroid-specific 5-aminolevulinic acid synthase (\textit{ALAS2}) gene. Affected males usually present in the first two decades of life with symptoms of anemia, and in middle age with manifestations of secondary iron overload. Phenotypic expression of XLSA varies considerably in males, and is partly related to the type of \textit{ALAS2} mutation, with more than 25 mutations described so far in more than 30 kindreds. In addition, modifying genes such as those for genetic hemochromatosis may significantly exacerbate XLSA in hemizygous males. On the other hand, genetic and acquired factors responsible for skewed X-chromosome inactivation may lead to late-onset XLSA in heterozygous females.

Whereas the existence of modifying genes capable of worsening XLSA is well established, little information is available on genetic factors that may attenuate or suppress its phenotypic expression. By studying a family with a new \textit{ALAS2} mutation, we provide evidence that phenotypic expression of XLSA may be absent in hemizygous males.
Study design

The proband (subject II-2 in Fig. 1A) was found to have microcytic anemia (Hb 7.6 g/dl; MCV 64 fL) with high RDW (30.9%) at the age of 38. Serum ferritin was raised, transferrin was 82% saturated and 30% of the patient’s bone marrow erythroblasts were ring sideroblasts. A diagnosis of XLSA was made. On treatment with 300 mg/d pyridoxine, hemoglobin level averaged around10 g/dL. Iron chelation therapy with deferoxamine (DFO) normalized serum ferritin.

The procedures followed were in accordance with the ethical standards of the institutional committee on human experimentation and with the Helsinki Declaration of 1975, as revised in 1983. Routine hematological measurements were obtained using a Bayer Technicon (Milan, Italy) H3 automatic cell analyzer. For evaluation of potential thalassemia defects, standard procedures were used. Molecular analyses of the HFE and $ALAS2$ genes were performed as previously described. The expression of mutant $ALAS2$ messenger RNA (mRNA) was evaluated in peripheral blood reticulocytes from the proband and his brother, as previously described.
Results and Discussion

The pedigree of the proband’s family is shown in Fig. 1A, while hematological and iron status data for the family members are reported in Table 1.

A single point mutation in exon 11 of the ALAS2 gene was found in the proband (Table 1 and Fig. 1C), after all exons, intron-exon boundaries, and the 5' and 3' flanking regions were sequenced in both directions. The only mutation found was a transition from G to A at nucleotide 1731 that predicts an amino acid change of a conserved arginine to the more compact histidine at position 560 (R560H). Two males (including the proband) were hemizygous, and five females, including the three obligate heterozygote daughters of the non-anemic hemizygous male (II-3), were heterozygous for this mutation (Table 1). These results were independently obtained in two different laboratories and the mutation was confirmed in a third. In addition, sequence analysis of cDNA derived from reticulocyte RNA revealed that both the proband and his brother expressed mRNA from the mutated ALAS2 allele in erythroid cells (Fig. 1D). The two brother’s DNA samples that were used for sequencing were shown to be non-identical and derived from the same parents by genotyping of informative short tandem repeat polymorphisms at 15 loci (data not shown).

In order to demonstrate that the above mutation was not a polymorphism, we studied 100 DNA samples from unrelated females, none of whom had this change. This indicates that the transition from G to A at nucleotide 1731 is not a polymorphism, since it was found in less than 1% of the population. In addition, significant difference was found between the mean RDW values (a phenotypic marker of XLSA) for five normal and five heterozygous females carrying the 1731G→A mutation. As detailed in Table 1 (footnote on RDW), these
latter had markedly higher RDW values (F=50, P=0.0001), indicating that all of them had the typical hematological phenotype of female carriers.

By contrast, the two hemizygous brothers had markedly different hematological phenotypes. The proband (II-2) showed a classical microcytic anemia with high RDW and secondary iron overload. In comparison, his brother (II-3) had normal red cell counts and no evidence of iron overload. However, he showed marginally low MCV and marginally elevated RDW on some occasions. A feature of XLSA is the presence of microcytic red cells. Fig. 1B shows red cell volume histograms of 3 representative family members: the non-anemic hemizygous brother shows an almost normal pattern.

Although unlikely, we could not exclude that the mutant ALAS2 in the non-anemic normal brother was silenced through a mechanism of intercistronic suppression.9 In order to establish whether other inherited disorders could explain the different phenotypes, additional studies were performed. Molecular analysis of HFE excluded the C282Y or H63D mutations, while investigations for beta or alpha thalassemia defects revealed no imbalance in globin chain synthesis in both brothers. No potentially causative non-genetic factors were identified.

Variable penetrance of pyridoxine-responsive XLSA has been previously reported. In a family with the mutation 1215C→G predicting an S388T amino acid change,10 three hemizygous males were studied. Their hemoglobin levels at clinical onset ranged from 5.0 to 14.4 g/dL, but all of them showed microcytosis (60-73 fL). Four hemizygous males were studied in a family with the mutation 923G→A predicting a G291S amino acid change,11 and variable hemoglobin levels at clinical onset were found. All patients, however, were anemic (hemoglobin from 9.2 to 12.5 g/dL) and showed reduced MCV values (60-74 fL).
Therefore, all these patients clearly had the XLSA phenotype, and modifying genes or acquired factors capable of worsening or ameliorating XLSA were likely playing a role in various individuals.

The present family is unique because the severe hematological phenotype of the proband was almost completely absent in his brother. The fact that all three of obligate heterozygote daughters of this non-anemic hemizygote had mild phenotypes (marginally elevated RDW), argues against worsening factors in these family members, and would be consistent with the presence of a silencing factor(s) in the nearly normal hemizygote, as in the case of complete suppression of congenital dyserythropoietic anemia type II in a homozygous male. On the other hand, different genetic backgrounds may be responsible for the expression of XLSA in the proband, similar to the variable clinical expression in heterozygous gene carriers for mutations in the autosomal dominant porphyrias.

Hemizygous males with ALAS2 mutations and absent XLSA phenotype may be less rare than thought. In fact, these individuals are likely to go unnoticed, since only males with abnormal phenotype are usually studied in families with X-linked inherited disorders. This emphasizes the importance of gene-based diagnosis in families with XLSA. Identification of the ALAS2 mutation in the subject II-3 has led to the recognition of a heterozygous state in his three daughters, who will now benefit from genetic counseling. In the future, they will be advised that their offspring might be at risk for anemia and iron overload but could benefit from a simple oral administration of pyridoxine that might prevent development of XLSA. We believe that all at-risk individuals in families with XLSA who request testing should have their DNA examined for ALAS2 mutations, irrespective of normal hematological findings, sex and age.
Acknowledgements

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Table 1. Hematological data and ALAS2 exon 11 sequence (from nucleotide 1725 to 1736).

<table>
<thead>
<tr>
<th>Subject</th>
<th>Sex/age</th>
<th>Hb</th>
<th>MCV</th>
<th>RDW*</th>
<th>Serum ferritin</th>
<th>ALAS2 paternal allele¶</th>
<th>ALAS2 maternal allele¶</th>
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<tr>
<td>Proband (II-2)</td>
<td></td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>- at clinical onset</td>
<td>M/38</td>
<td>7.6</td>
<td>64</td>
<td>30.9</td>
<td>859</td>
<td>-</td>
<td>GTCGCCATCCTG</td>
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<tr>
<td>- under treatment</td>
<td>M/42</td>
<td>10.1</td>
<td>69</td>
<td>25.9</td>
<td>142</td>
<td>-</td>
<td>GTCGCCATCCTG</td>
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<tr>
<td>Subject II-3</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
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</tr>
<tr>
<td>M/36</td>
<td>14.1</td>
<td>83</td>
<td>14.2</td>
<td>124</td>
<td>-</td>
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<td>M/37</td>
<td>14.7</td>
<td>85</td>
<td>13.7</td>
<td>ND</td>
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<tr>
<td>M/40</td>
<td>13.7</td>
<td>82</td>
<td>13.9</td>
<td>110</td>
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<td>Subject II-4</td>
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<td>13.1</td>
<td>83</td>
<td>15.5</td>
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<td>GTCGCCGT CCTG</td>
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<td>- III-5</td>
<td>F/17</td>
<td>12.1</td>
<td>86</td>
<td>14.1</td>
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<td>GTCGCCGT CCTG</td>
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<td>77</td>
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<td>14.1</td>
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<td></td>
<td>12.0-16.0</td>
<td>83-97</td>
<td>11-14</td>
<td>12-150</td>
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<tr>
<td>- adult males</td>
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<td>83-97</td>
<td>11-14</td>
<td>20-250</td>
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<td>75-95</td>
<td>11-14</td>
<td>10-50</td>
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</table>

* RDW = Red-cell Distribution Width, i.e., the standard deviation of the mean red-cell volume expressed as percentage of the mean red-cell volume. The RDW values of the five heterozygous women (II-4, III-5, III-7, III-8, III-10) ranged from 14.1 to 15.5% (mean ± SD: 14.6% ± 0.6%) while those from five normal female family members ranged from 12.0 to 13.0% (mean ± SD: 12.1% ± 0.4%).

¶ Wild-type sequence: GTCGCCGT CCTG. Mutations are shown in **bold**.

ND = not determined.
Fig. 1
Legend to Figure

Fig. 1. Most relevant clinical and molecular findings in the family studied.

A. Pedigree of the family studied. Circles denote female family members, squares male family members, and diamonds additional members of either sex (the number of additional members is shown in the diamonds); symbols with diagonal lines indicate deceased members. Solid squares indicate hemizygous males, while half-solid circles indicate heterozygous women.

B. Red cell volume histograms obtained with a Bayer/Technicon H3 in three family members. The vertical tick marks correspond to 10 fL increments. Subject II-7 shows a typical normal picture. Subject II-4 is a typical heterozygous woman with a small proportion of microcytic red cells (tail on the left side of the histogram). The non-anemic hemizygous brother (subject II-3) shows a red cell volume histogram that is almost normal.

C. Dideoxy dye terminator sequence analysis of PCR-amplified, exon 11 genomic DNA from a normal individual (NL), the proband (II-2) and his brother (II-3).

D. Sequence analysis of reverse transcribed ALAS2 mRNA expressed in reticulocytes from the anemic (II-2) and non-anemic (II-3) hemizygous brothers.
References


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