A novel mutation in exon 5 of the ALAS2 gene results in X-linked sideroblastic anemia

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Abstract

\textbf{Background:} Mutations in the erythroid-specific 5-aminolevulinate-synthase gene (ALAS2) have been identified in many cases of X-linked sideroblastic anemia (XLSA). \textbf{Methods:} A polymerase chain reaction-mediated restriction fragment length polymorphism (RFLP) assay was used. \textbf{Results:} A G527T point mutation was identified. This resulted in a substitution of tyrosine for asparagine at residue 159 (D159Y). This mutation was also identified in the mother of the two probands. Mutations in all three individuals were confirmed by DNA sequencing analysis. \textbf{Conclusions:} We identified a missense mutation in exon 5 of the ALAS2 gene in two brothers of a consanguineous marriage, who were clinically pyridoxine-responsive. © 2002 Published by Elsevier Science B.V.

Keywords: ALAS2; X-linked sideroblastic anemia; Mutation analysis

1. Introduction

X-linked sideroblastic anemia (XLSA) is the most common form of inherited sideroblastic anemia [1]. It is a clinically heterogenous disorder characterized by ineffective erythropoiesis with hypochromic, microcytic anemia, splenomegaly, elevated tissue and serum iron, and ringed sideroblasts in the bone marrow [2]. It predominately affects males in infancy or early childhood. Females may also be affected by lyonization of the X chromosome. The 5-aminolevulinate-synthase gene (ALAS2) has been localized to Xp11.21 and mutations in this gene have been associated with anemia [3]. ALAS is the first and rate-limiting enzyme involved in heme biosynthesis and requires pyridoxal 5'-phosphate (PLP) as a cofactor. PLP is produced from pyridoxine [4,5]. Isoenzymes of ALAS include both erythroid specific (ALAS-E), which is restricted to erythroid cells, and nonspecific (ALAS-N) forms [6,7]. The defective activity of this enzyme in patients with XLSA leads to decreased heme syn-
thesis with diminished protoporphyrin IX, decreased hemoglobin concentrations, and increased tissue iron. This disorder is subdivided into pyridoxine-responsive and pyridoxine-refractory types based on clinical responsiveness to therapy with pyridoxine [8]. While mutations have been described that result in loss of function of the ALAS, there have been other reports of gene mutations which result in a responsive form of \( \text{ALAS2} \) deficiency [9,10]. Greater than 15 mutations in the \( \text{ALAS2} \) gene have been described [11–13]. In this report, we demonstrate a missense mutation in exon 5 of the \( \text{ALAS2} \) gene in a case of familial XLSA.

2. Materials and methods

2.1. Patients

The probands are two brothers, the result of a consanguineous marriage of first cousins. JS, born in 1985, was first seen by the Division of Pediatric Hematology at the University of Connecticut Health Center at age 3 1/2 years. JS has multiple congenital anomalies including pelvic kidney, glomerulopathy, dysmorphic facial features, clubfoot, seizure disorder, and developmental delay. His initial evaluation revealed a hemoglobin of 7.3 g/dl, a hematocrit of 22.5%, a mean corpuscular volume (MCV) of 53.8 fl, a red cell distribution width (RDW) of 26.3%, a reticulocyte count of 0.4–2.6%, serum iron of 164 µg/dl, total iron binding capacity (TIBC) of 276 µg/dl, a serum ferritin of 87 mg/dl, and free erythrocyte protoporphyrin of 22 µg/dl. A peripheral smear revealed hypochromic microcytic RBCs. Hemoglobin electrophoresis, hemoglobin \( \alpha \) gene mapping, and cytogenetic studies were normal. A bone marrow aspirate revealed a hypercellular bone marrow with erythroid hyperplasia. The erythroid precursors appeared normoblastic with occasional dyserythropoietic and poorly hemoglobinized forms. Sideroblasts and ringed sideroblasts were identified and comprised < 10% of all erythroid precursors (Fig. 1). A bone marrow evaluation revealed a hypercellular marrow with erythroid hyperplasia. The erythroid precursors appeared normoblastic with occasional dyserythropoietic and poorly hemoglobinized forms. Sideroblasts and ringed sideroblasts were identified and comprised < 10% of all erythroid precursors (Fig. 1, inset). He was started on pyridoxine (250 mg/d) and returned 6 weeks later with a hemoglobin of 11.3 g/dl, hematocrit of 38.1%, and an MCV of 68.9 fl.

The parents of these boys are first cousins. The mother suffered a stroke at age 25 years and has a seizure disorder. Her CBC revealed a hemoglobin of 12.7 g/dl, hematocrit of 37.1%, and MCV of 89.1 fl. The father’s CBC revealed a hemoglobin of 15.1 g/dl, a hematocrit of 45.1%, and an MCV of 84 fl. In order to confirm the suspected diagnosis of X-linked sideroblastic anemia in this family, molecular studies were performed on the \( \text{ALAS2} \) gene.

2.2. Morphology

Bone marrow aspirates and clot sections were studied on both children. Bone marrow aspirates were air-dried and stained with Wright stain and Prussian blue stain.

2.3. Molecular pathology

Peripheral blood samples were obtained in EDTA-anticoagulated tubes and sent to the laboratory for analysis of the \( \text{ALAS2} \) gene. As part of the differential diagnosis and due to pyridoxine-responsiveness, molecular studies were carried out to confirm the diagnosis of sideroblastic anemia. DNA was extracted from peripheral blood leukocytes using the Puregene...
extraction kit (Gentra, Systems, Minneapolis, MN) according to the recommendations of the manufacturer.

Initially, restriction fragment length polymorphism (RFLP) analysis was used to screen for previously published mutations. Genomic DNA (0.5–1.0 μg), isolated as described above, was incubated in a total reaction volume of 50 μl containing 40 ng of both the forward and reverse gene specific primers, 2.0 U AmpliTaq Gold (Perkin-Elmer, Norwalk, CT), 200 μmol/l each deoxynucleotide triphosphate, 1.5 mmol/l MgCl₂, 10 mmol/l Tris–HCl (pH 8.3), 50 mmol/l KCl, and 0.001% gelatin. The primer sequences were described by Cotter et al. [11]. PCR amplification was performed in the Gene Amp PCR System 9700 thermocycler (Perkin-Elmer) using the following conditions: initial denaturing at 94 °C for 12 min followed by 35 cycles of 30 s denaturing at 94 °C, 45 s annealing at 60 °C, and 30 s extension at 72 °C. The final cycle included a 3-min extension step at 72 °C. Following amplification, a restriction enzyme digestion was performed to detect the ALAS2 exon 5 mutation. The digest was performed in a total volume of 50 μl and consisted of 30 μl of PCR product, 14 μl of dH₂O, 1 μl of Sau3AI restriction enzyme (New England BioLabs, Beverly, MA), 5 μl 10 × buffer. Samples were then incubated overnight at 37 °C. Restriction fragment size analysis was performed by visualization of digested PCR products after separation by gel electrophoresis in a 10% polyacrylamide gel. The gel was stained with ethidium bromide and examined under UV light.

Exon 5 DNA Sequencing

Sau3AI restriction recognition sequence: gate

Normal ALAS2
481 cagttatgcacagtcttcagggcaagaatcagagaaa caggatcac acc 540

LS and JS ALAS2
481 cagttatgcacagtcttcagggcaagaatcagagaaa cagtacct acc 540

Mother ALAS2
481 cagttatgcacagtcttcagggcaagaatcagagaaa cag g/Tate ac acc 540

Fig. 2. DNA sequence analysis data of the normal ALAS2 exon 5, patients’ (LS and JS) mutant ALAS2 exon 5, and heterozygous mutant ALAS2 exon 5 from the patients’ mother. Note the Sau3AI restriction enzyme recognition site (underlined).
using the Stratagene Eagle Eye II documentation system.

To confirm RFLP findings, PCR products were also purified using the QIAquick PCR Purification kit (Qiagen, Valencia, CA) and sequenced by using the BigDye Terminator Cycle Sequencing kit (Perkin-Elmer/Applied Biosystems, Foster City, CA). The fluorescence-labeled cycle sequencing products were purified by using the DyeEx Spin kit (Qiagen) and DNA sequences were determined on an ABI PRISM 310 Genetic Analyzer (Perkin-Elmer).

3. Results and discussion

In 1945, Cooley [14] first reported several cases of X-linked sideroblastic anemia describing “a severe type of hereditary anemia with elliptocytosis.” Since Cooley’s initial description, to date, there have been more than 15 described mutations of the erythroid-specific ALAS2 gene. Point mutations have been described in the promoter regions and missense mutations in the coding regions of the ALAS2 gene. De novo mutations account for 29% of all described mutations and are found in CpG dinucleotides resulting from spontaneous deamination of 5-methylcytosine to thymine [9]. The mutation identified in this family is not a de novo occurring mutation and does not occur at a CpG dinucleotide. The most common ALAS2 gene mutations are found in exon 9 and exon 5 [9].

Both pyridoxine-refractory and -responsive XLSA have been reported. While ALAS2 mutations were initially thought to result in pyridoxine-refractory XLSA, we and others report individuals with ALAS2 mutations that are pyridoxine-responsive [1,10]. It is possible that rates of proteolytic processing, alterations in pyridoxal 5‘-phosphate binding sites, and amino acid substitutions do not significantly alter ALAS activity [10].

The mutation found in the two brothers and mother in this case was a single point mutation in exon 5 of the ALAS2 gene (Fig. 2). This mutation resulted in a G to T transversion at nucleotide 527 with the substitution of tyrosine for asparagine at residue 159 (D159Y). This point mutation destroys a Sau3AI restriction site that is detected by PCR-mediated RFLP analysis. To the best of our knowledge, this is the first reported case of XLSA associated with this mutation. These findings and the presence of varied clinical and hematologic manifestations in heterozygous carriers with XLSA emphasize the importance of molecular diagnostics as a means of confirming this diagnosis.

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