Red Cell Disorders

Onset of X-linked sideroblastic anemia in the fourth decade

We report the case of a 40-year female who manifested late onset, pyridoxine-refractory X-linked sideroblastic anemia, heterozygous for the first described frameshift *ALAS2* mutation, *CD506-507 (-C)*. On presentation she had macrocytic anemia with severe iron overload.

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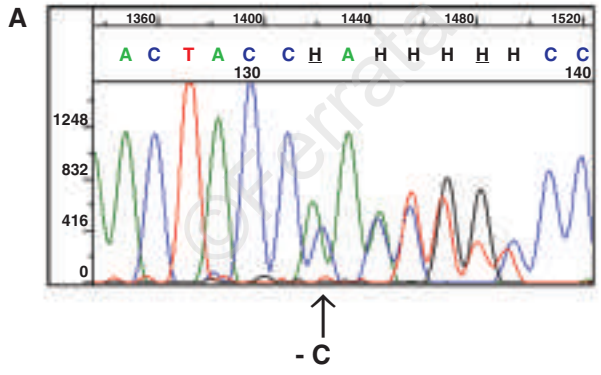
X-linked sideroblastic anemia (XLSA), the most common inherited form of sideroblastic anemia, is associated with several mutations (primarily missense) in the erythroid-specific 5-aminolevulinic acid synthase gene, *ALAS2*, located on chromosome Xp11.21. In XLSA cases due to missense mutations the anemia usually improves with pyridoxine supplementation.[1]

In the majority of females heterozygous for *ALAS2* mutations, red blood cell (RBC) progenitors with the wild-type *ALAS2* allele are sufficient to sustain a normal level of RBC production[2-4] and a normal (Hb) hemoglobin concentration, despite dimorphic red cell morphology. Conversely, as in any X-linked disorder, the phenotype of female carriers may be influenced by unbalanced X-chromosome inactivation (Lyonization),[5,6] resulting in anemia of different severities.[7]

A 41-year old Portuguese Caucasian female was referred to our Department for investigation of severe macrocytic anemia with significant iron overload. She had previously been healthy, with no history of drinking or drug abuse. During pregnancies she had been prescribed oral iron; in her last pregnancy (9 years previously) Hb was 101 g/L and mean cell volume (MCV) 96 fL. She reported a recent history of asthenia, mild breathlessness, amenorrhea and skin hyperpigmentation. Her spleen was not palpable and the liver border was just below the costal margin.

Laboratory data: Hb 71 g/L, MCV 107 fL, mean cell hemoglobin 36 pg, mean cell hemoglobin concentration 250 g/L, red cell distribution width 22%, reticulocytes 2%, serum ferritin 3974 µg/L (N 14-150 µg/L), total bilirubin 7.1µmol/L (N <17), aspartate aminotransferase 54 IU/L (N 5-35 IU/L), alanine aminotransferase 69 IU/L (N 5-35 IU/L), lactate dehydrogenase 175 IU/L (N 211-423 IU/L), and homocysteine 7.9 µmol/L (N 5-10). White cell and platelet counts and morphology were normal. The peripheral blood smear showed anisopoikilocytosis and dimorphism with two distinct red cells populations, one normochromic and the other hypochromic and microcytic. Bone marrow examination revealed dyserythropoiesis with megaloblastoid features and 20% of ringed sideroblasts. Liver biopsy showed increased stainable iron; quantification of liver iron by magnetic resonance techniques found more than 350 mmol/g of hepatic tissue. Screening for *HFE* gene mutations associated with hemochromatosis revealed only *H63D* heterozygosis. Amplification and sequencing of *ALAS2* gene 11 exons with flanking intronic sequence (50 to 100 nt), 250 base pair (bp) pro-



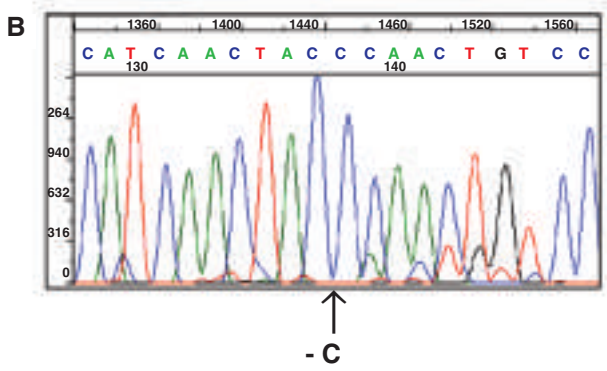


Figure 1 A. *ALAS2* gene DNA sequencing showing (-C) deletion on CD506-507 leading to a frameshift. B. *ALAS2* cDNA sequencing showing the wild type and the mutated transcripts. In spite of being more active, the mutated allele is less represented, probably because of instability of the abnormal mRNA. *ALAS2* mRNA was isolated from peripheral blood reticulocytes with the MicroPoly (A)Pure™ (Ambion®, USA) kit and reverse transcribed into cDNA.

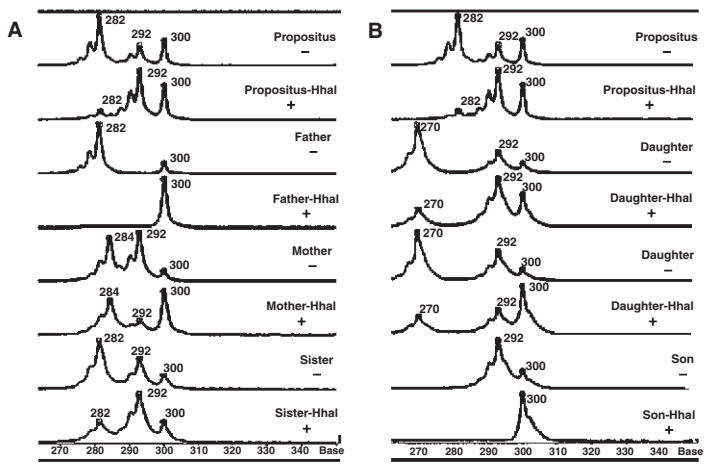


Figure 2. Hematopoiesis clonal analysis with *HUMARA* assay performed in DNA from peripheral blood leukocytes, as previously described[5] with minor modifications. The – and + signs indicate sample undigested (-) or digested (+) by the methylation-sensitive restriction endonuclease Hhal. A. The propositus and her sister inherited the same paternal alleles (282 and 292). In Hhal digested DNA the paternally-derived allele (282) is less amplified than the maternal one (292), indicating that the paternally-derived *HUMARA* allele is less methylated (more active) than the maternal. B. The three offspring inherited the 292 maternal allele. The fact that none of the *propositus*' family member have the *ALAS2 CD506-507 (-C)* mutation indicates that it occurred *de novo* on the same chromosome as the 282 paternally-derived allele.

moter region, the 5'UTR and 220 bp of the 3' flanking sequence, were accomplished as previously described[8] with minor changes. The only difference from the normal published sequence was a nucleotide deletion *CD506-507(-C)* in the heterozygous state (Figure 1A). This frameshift mutation, not previously described, is expected to lead to a shortened polypeptide chain (truncated by 60 amino acids at the C-terminal end).

Analysis of X chromosome inactivation showed heterozygosis for the HUMARA loci polymorphisms. The HUMARA cleavage ratio between alleles was 15.0, indicating an extremely skewed Lyonization of 94:6 according to the equation reported by Aivado *et al.*[9] Screening for the C→G mutation at position -43 in the *XIST* minimal promoter region was negative.[10] *ALAS2* mRNA studies showed the wild type and mutated transcripts (Figure 1B), although the mutated transcript was less represented, probably because of its instability. We were able to carry out a family study. The patient's father, mother, 2 brothers, 1 sister, 2 daughters and 1 son had normal hematologic parameters and screening for the *ALAS2* mutation was negative. Analysis of HUMARA loci polymorphisms established that the *ALAS2 CD506-507(-C)* mutation had occurred *de novo* in the allele inherited from the father and the patient's offspring had not inherited the mutated allele (Figure 2). Analysis of DNA extracted from buccal cells also showed heterozygosis for *ALAS2 CD506-507(-C)*, indicating that this is a somatic mutation. The patient was treated with oral pyridoxine (300 mg/day) for several months with no hematologic improvement and started iron chelation therapy with subcutaneous desferroxamine (1000 mg/day five days a week) and tea ingestion. Folic acid (10 mg/day) and hydroxocobalamin (30000 μg i.m.) administration did not decrease the macrocytosis. The patient's Hb level remained stable for 9 months with no major symptoms or signs of anemia; the spleen became palpable and ferritin level decreased to 900 μg/L. In the following months her Hb level slowly decreased, congestive heart failure was noted and a regular transfusion program was started. She recently restarted regular menstruation.

In summary, we describe the late onset of pyridoxine-refractory X-linked sideroblastic anemia in a female heterozygous for a newly described frameshift mutation in exon 10 of the *ALAS2* gene, which occurred *de novo* in her father's allele. This mutation is predicted to lead to a non-functional truncated polypeptide, explaining the failure to respond to pyridoxine treatment. The patient's clinical history suggests that during the first three decades of life her erythroid precursors were able to produce enough ALAS-e to maintain fairly normal erythropoiesis but that in the fourth decade, an increased skewed Lyonization in favor of the mutated allele caused ineffective erythropoiesis leading to severe anemia. Fortunately, her children did not inherit the mutated allele.

This case emphasizes the need to screen for *ALAS2* mutations in female patients with anemia and ringed sideroblasts irrespective of age and the presence of macrocytic red cells, provided platelets and WBC appear normal. Mutation screening is indicated in members of these families, for genetic counselling and early prevention of iron overload.

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Chronic Myeloproliferative Disorders

Lack of response to imatinib mesylate in a patient with accelerated phase myeloproliferative disorder with rearrangement of the platelet-derived growth factor receptor β-gene

Imatinib mesylate has been reported to produce positive results in atypical chronic myeloproliferative disorders (CMD) with chromosomal translocations that disrupt the platelet-derived growth factor receptor β gene (*PDGFRB*). We used imatinib to treat a 49-year old man with atypical CMD in accelerated phase and the *H4 (D10S170)-PDGFRB* fusion gene. After 3 months of treatment, we observed grade 4 hematologic toxicity and a lack of response.

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In September 1999, a 49-year old man presented with asthenia and huge splenomegaly. Blood counts performed 2 years previously had already shown hyperleukocytosis and eosinophilia that were not investigated. The blood count at presentation showed hemoglobin 68 g/L, white blood cell count (WBC) of 7.4×10^9/L with 64% neutrophils, 19% lymphocytes, 6% monocytes, 6% eosinophils, 1% basophils, 2% metamyelocytes, 1% myelocytes, 1% promyelocytes, 1% erythroblasts and a platelet count of 63×10^9/L. Bone marrow aspiration was difficult and showed granulocytic hyperplasia without excess of blast cells and few megakaryocytes. The bone marrow biopsy, stained with hematoxylin-eosin and May-Grünwald Giemsa, showed granulocytic hyperplasia, established myelofibrosis (grade III reticulin) and no evidence of blastic transformation. Cytogenetic analysis on bone marrow cells showed: 46,XY, t(5;10) (q33;q21) [18] /46,XY [2]. Reverse transcription polymerase chain reaction (RT-PCR) did not detect *BCR-ABL* transcripts. RT-PCR to detect the *H4-PDGFRB* fusion gene was performed on bone marrow cells as previously described.[1] The fusion junction was identical to that found in the two previously reported cases (Figure 1). The patient was given hydroxyurea which controlled the leukocytosis but the anemia and thrombocytopenia worsened. The spleen enlarged and a splenectomy was performed. The *in vitro* sensitivity of mononuclear (MN) cells to imatinib was studied. Bone marrow MN cells were cultured as previously reported[2] and compared to CML cells. The viability of MN cells exposed or not to 1 μM of imatinib was determined and found to be 50% versus 70% at day 2 and 35% versus 55% at day 3. It should, however, be noted that 100% of cells were dead by the 6th day of culture in the presence or absence of imatinib (Figure 2). In February 2002, the patient began therapy with imatinib mesylate at 400 mg daily. At this time cytogenetics analysis showed additional abnormalities of t(5;10) in 2 mitoses: add(3)(p21) and monosomy 15.

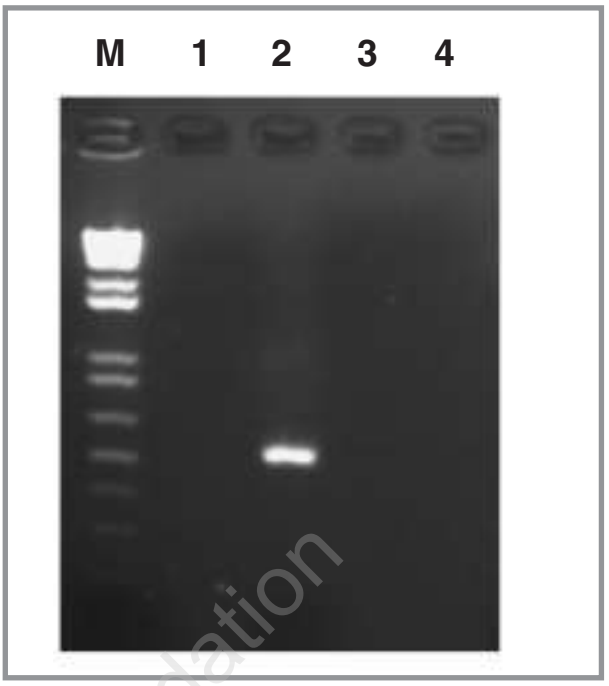

Figure 1. RT-PCR analysis to detect the *H4-PDGFRB* fusion. M is the 1kb+ marker (Invitrogen), lanes 1 and 3 represent negative control patients, lane 2 represents the t(5;10) patient and lane 4 is a negative PCR control.

Imatinib was stopped after 3 months of therapy because of severe hematologic toxicity with the patient requiring platelet and red blood cell transfusions. In the six months following discontinuation of imatinib, hematologic recovery was observed. A second trial of imatinib immediately led to a worsening of thrombocytopenia. Imatinib was definitively stopped and blood counts again recovered. Cytogenetic analysis, repeated after each course of imatinib, showed the persistence of t(5;10) in all the mitoses and *H4-PDGFRB* transcript was still detectable by RT-PCR. Hydroxyurea was subsequently given with persisting good hematologic control until now.

Abnormal activation of *PDGFRB* was first described as a consequence of the t(5;12)(q33;p13), which fuses the 5'end of *ETV6* to the 3'end of *PDGFRB* including the entire tyrosine kinase domain,[3] and complete and durable responses to Imatinib were reported in four patients with t(5;12) translocation.[2] Other translocations involving the same region of *PDGFRB* have been reported:[4] t(5;10) (q33;q21) translocation fusing *PDGFRB* to *H4(D10S170)*, a gene encoding for a 585-amino acid protein with no significant homology to known genes and with unknown function, has been reported in 3 patients.[5,6,7] *H4* is fused to the *ret* gene as a result of an inv(10)(q22q21) in a subset of papillary thyroid carcinomas. The H4-ret fusion protein is a constitutively active tyrosine