Pyridoxine-refractory congenital sideroblastic anaemia with evidence for autosomal inheritance: exclusion of linkage to ALAS2 at Xp11.21 by polymorphism analysis

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Abstract
A son and daughter of unaffected parents had transfusion dependent, pyridoxine-refractory sideroblastic anaemia from birth. Their haemoglobin levels were 4·3 and 6·4 g/dl, respectively. δ-Aminolaevulinate synthase activity in erythroblasts from fractionalized marrow of the sister was 135 pmol δ-aminolaevulinate formed/10^6 erythroblasts/hour (normal range 110–650 pmol). While mutations of the erythroid-specific δ-aminolaevulinate synthase gene (ALAS2) at Xp11.21 have been reported in patients with X linked sideroblastic anaemia, sequence analysis of the ALAS2 gene in the son did not identify any mutations in the coding region, the intron/exon boundaries, or the 1 kb 3′ promoter region. A useful polymorphism was found in the 3′ region of the ALAS2 gene, a G to A transition, 220 nt 3′ of the AATAAA polyadenylation signal. Mismatch PCR at this site and subsequent discrimination by XmnI restriction analysis of 148 alleles identified the gene frequency of this polymorphism to be 25%. Analysis of the inheritance of this intragenic polymorphism showed that the affected sibs received different maternal alleles at the ALAS2 locus, excluding mutations in this gene as the cause of their sideroblastic anaemia. Furthermore, the absence of a dimorphic erythrocyte population in the mother, coupled with the demonstration of random X inactivation in her peripheral leucocytes, showed that the mother was not the carrier of any X linked sideroblastic anaemia mutation. These results strongly suggest that the sideroblastic anaemia in this family is an autosomal recessive trait.

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Case reports

CASE 1
This male infant, born 12.6.89, the first child of healthy unrelated parents of English descent, was pale at birth. At 2 days of age there was respiratory distress, hepatosplenomegaly, and scrotal oedema. Investigation of the proband showed a hypochromic, microcytic anaemia (haemoglobin 4.3 g/dl, MCV 68 fl, platelets 241 x 10^11/l, white cell count 10.2 x 10^9/l). Haemoglobin electrophoresis was normal. The bone marrow was hyperplastic with many late normoblasts. Perl's stain showed increased storage iron with at least 60% ringed sideroblasts. No vacuolisation of erythroid or myeloid marrow precursors was observed. At 7 months of age pyridoxine therapy (100 mg/day orally) was started. There was no response to this nor to a subsequent three months' treatment with pyridoxal 5'-phosphate (100 mg/day orally). Blood transfusions were required every five to six weeks from birth because of symptomatic anaemia. Iron chelation therapy (subcutaneous deferoxamine) was started at 2 years of age. There was no clinical evidence of malabsorption. At the age of 3-5 years, growth and development were normal, pretransfusion haemoglobin was 6-5 g/dl and serum ferritin 318 ng/ml (normal range 17-280). Haemoglobin and red cell indices were normal in both parents and a maternal grandmother and aunt, with no evidence of red cell dimorphism.

CASE 2
This younger sister of the proband, born 14.6.91, has followed a similar course to that of her brother. At birth there was pallor, oedema, and hepatosplenomegaly. Haemoglobin was 6.4 g/dl, MCV 62 fl, platelets 430 x 10^11/l, and white cell count 12.1 x 10^9/l. Blood transfusions were required every four weeks from birth. There was no response to three months' oral therapy with pyridoxine (100 mg/day) started at 1 month of age or three months' subsequent treatment with pyridoxal 5'-phosphate (100 mg/day). The karyotype was 46,XX. Bone marrow aspiration at the age of 7 months, after six blood transfusions, showed scanty erythroblasts with marked sideroblastic features. At the age of 2-5 years, growth and development were normal. Pretransfusion haemoglobin was 6.0 g/dl and serum ferritin 260 ng/ml. Desferrioxamine treatment has not yet been started.

Materials and methods

ERYTHROBLAST FRACTIONATION
Bone marrow myeloid cells were lysed by a specific IgM monoclonal antibody (TG1). Remaining erythroblasts were fractionated using Percoll equilibrium density centrifugation to provide an erythroid fraction enriched for early and intermediate erythroblasts.

ALAS ACTIVITY
ALAS activity was assayed in whole and fractionated marrow by a radiochemical method using high performance liquid chromatography to isolate [3H]-aminolaevulinic acid (ALA), as previously described. ALA utilisation was completely inhibited by 2 mmol/l EDTA and 0.1 mmol/l succinyl acetone. Succinyl coenzyme A generation was provided by 0.5 units succinate thiokinase, 5 mmol/l GTP, 1.35 mmol/l coenzyme A, and 2 mmol/l MgCl2. Results were expressed as pmol ALA synthesised in one hour per 10^6 erythroblasts.

MUTATION ANALYSIS
Screening for mutations in the X chromosomal ALAS2 gene was accomplished by sequence analysis as previously described. Genomic DNA was obtained from white cells of case 1 by phenol/chloroform extraction of EDTA blood using standard methods and all 11 exons, including the intron/exon boundary regions, 1 kb of sequence 5' of the first exon, and 350 bp 3' of the last exon, were PCR amplified, subcloned into pGEM4Z (Promega) and sequenced. Oligonucleotides -5'-GGCAGACACAACGGCAGATCC3' (sense) and -5'-GGAATTCTTATTTAACATCTTTTGTG-3' (antisense) were synthesised on a model 380B DNA Synthesiser (Applied Biosystems) as previously described and used to amplify a 353 nt polymorphic fragment from the 3' region of the ALAS2 gene. The antisense primer was designed for mismatch PCR, where the underlined nucleotide differed from the complement of the normal genomic sequence (...AGC-3'). The resultant PCR product from the DNA of case 1 contained an XmnI site, whereas DNA from most normal subjects did not. Mismatch PCR was performed on 148 alleles from 81 unrelated subjects (67 females and 14 males). Each amplification reaction contained 1 ng of genomic DNA, 100 pg of each primer, 50 mmol/l KCl, 10 mmol/l Tris-HCl, pH 9.0, 0.1% Triton X-100, 1.5 mmol/l MgCl2, and 2.5 U Taq polymerase (Promega) in a 100 µl reaction volume. Conditions were 94°C for four minutes, then 30 cycles of 94°C for 30 seconds, 62°C for 30 seconds, and 72°C for one minute. PCR products were digested with XmnI (New England Biolabs), electrophoresed in a 1.5% agarose gel, and visualised under UV light after ethidium bromide staining.

X INACTIVATION ANALYSIS
DNA was extracted from peripheral leucocytes of both patients and their parents, double digested with the restriction enzymes PsuI/MspI and PsuI/HpaII and the DNA fragments separated by electrophoresis in 0.8% agarose. Fragments were blotted onto nylon membranes (Hybond N, Amersham Ltd) by capillary transfer and the membranes hybridised with the probe M27PB which had been radiolabelled with [32P]dCTP by the random priming extension method (Amersham).
Figure 1  Sequence comparison of the normal and polymorphic alleles from the 3' region of the ALAS2 gene. The G to A transition identified in case 1 is indicated by asterisks. For additional details see Materials and methods.

Results

ALA SYNTHASE ANALYSSES

Measurement of bone marrow ALAS enzymatic activity was carried out to assess the possible involvement of this enzyme in the pathogenesis of sideroblastic anaemia in this family. ALAS activity in unfractonated marrow from case 2 was somewhat reduced at 370 pmol ALA formed/10^6 erythroblasts/hour (normal range 500–1200). Enzyme activity in the erythroblast fraction was 135 pmol ALA formed/10^6 erythroblasts/hour, in the low end of the normal range (110–650).

Sequence analysis of the ALAS2 gene showed that the mutant sequence from case 1 carried no mutation within any exon, within the intron-exon boundaries, or within 1 kb of sequence 5' to exon 1. However, a single nucleotide change (a G to A transition) was identified in the 3' region of the gene, 220 nucleotides 3' of the AATAAA polyadenylation signal. To identify this mutation easily, a synthetic mismatch PCR primer was constructed such that this nucleotide change would result in the creation of an XmnI restriction site in the PCR product (fig 2). XmnI digestion of the mutant product generated fragments of 323 and 30 nucleotides, while the normal uncut product was 353 nt. Screening 148 alleles from 81 unrelated subjects (67 females and 14 males) by mismatch PCR and XmnI digestion identified 111 alleles with the wild type nucleotide G, and 37 alleles with the mutation phenotype, indicating that the nucleotide change was a polymorphism with a frequency of 25%. Screening the patients' family for the XmnI polymorphism confirmed that case 1 was XmnI positive and showed that the father and affected daughter (case 2) were XmnI negative while the mother was heterozygous (fig 3). Thus ALAS2 mutations were confirmed not to be the cause of X linked sideroblastic anaemia in this family since the affected sibs inherited different ALAS2 alleles from the unaffected mother.

METHYLATION PATTERN ANALYSIS

If the proband’s mutation was X linked one would have expected to see a dimorphic blood film in the mother, unless Lyonisation was non-random. Since the mother’s blood film was normal, a methylation analysis was carried out to assess Lyonisation status. Methylation analysis of leucocyte DNA was conducted with the polymorphic probe M27β as shown in fig 4. Lanes 1, 3, 5, and 7 were PstI/MspI digests and lanes 2, 4, and 6 and 8 were PstI/HpaII digests. Using the methylation-insensitive MspI, the mother (lane 3) was clearly heterozygous (alleles 1 and 2) and the father (lane 1) was hemizygous for a smaller fragment (allele 3). At this locus the affected son and daughter (lanes 5 and 7) have inherited opposite alleles from their mother (alleles 2 and 1, respectively). Using the methylation-sensitive HpaII,
the active, methylated X chromosome from the proband (lane 6) and his father (lane 2) was uncut at the Mspl sites identified in lanes 1 and 5. Two darker bands corresponding to methylated alleles 1 and 2 and two lighter bands corresponding to unmethylated alleles 1 and 2 were visible in the mother’s DNA (lane 4). This excess of methylated over unmethylated alleles in peripheral leucocytes has been shown previously; while all of the active X allele is methylated at the CCGG site, up to 60% of the inactive X allele can also be methylated.23 Thus, though reduced in intensity, the ratio of the HpaII cut (lower) bands provides an accurate estimation of the ratio of the inactive alleles. From the HpaII cut bands in fig 4, lane 4, the mother’s DNA showed approximately 50/50 random X inactivation. Since the mother’s blood smear was not dimorphic and Lyonisation was not severely skewed, this result extends the exclusion of ALAS2 as a possible cause of sideroblastic anaemia in this family to exclusion of the X chromosome as the carrier of the mutation.

Discussion
The molecular events causing the various inherited sideroblastic anaemias are beginning to be defined. Recently, hypochromic, microcytic, pyridoxine-responsive X linked sideroblastic anaemia (XLSA) was shown to be caused by point mutations in the erythroid-specific ALAS2 gene.30-31 In addition, a macrocytic sideroblastic anaemia with vacuolated marrow precursor cells and pancreatic insufficiency was shown to be caused by mitochondrial DNA deletions.32 Since one of the family members studied in this report had somewhat reduced marrow erythroblast ALAS activity, the family was evaluated for mutations in the ALAS2 gene.

The ALAS2 gene of the proband was normal by sequence analysis of the coding region, intron-exon junctions, and 5’ and 3’ flanking regions. This provided evidence for autosomal inheritance since mutations within this locus are the only described molecular defects in XLSA.30-31 The discovery of an informative polymorphic nucleotide change in the 3’ end of the ALAS2 gene enabled conclusive demon-

stration that the erythroid-specific ALAS2 gene was not involved in the pathogenesis of sideroblastic anaemia in this family. The proband, case 1, was positive for the XmnI polymorphism while his affected sister, case 2, was homozygous for the wild type allele. Thus, they have clearly inherited different ALAS2 alleles from their unaffected mother, ruling out a defective ALAS2 gene as the cause of sideroblastic anaemia in this family. The slight reduction in ALAS activity observed in case 2 may be a secondary consequence of iron overload in the mitochondria.

In addition to XLSA caused by ALAS2 mutations, carrier females for X linked sideroblastic anaemia associated with ataxia also have a dimorphic erythrocyte population.34 Methylation studies were undertaken to determine whether the expression of sideroblastic anaemia in this family could be explained by the presence of some X linked gene and the non-random inactivation of the normal X chromosome. The polymorphic probe M27β and Mspl digestion is highly informative with greater than 90% heterozygosity in females.35 Unlike the unmethylated CpG rich islands 5’ of active housekeeping genes, certain X sites in the M27β region are methylated on the active chromosome and unmethylated on the inactive chromosome.36 These sites occur within the extensively methylated 5’ region of a LINE-1 repetitive element at the DXS255 locus.37 The X inactivation status of alleles may be determined after digestion with the methylation-sensitive restriction enzyme HpaII. Although X inactivation status was evaluated in the patients’ leucocytes rather than erythroblasts, previous studies have shown that both erythroid and myeloid cells have the same isozyme ratios for X linked genes.38 After HpaII digestion, it was clear that the mother’s X chromosomes were randomly inactivated as judged by the appearance of two HpaII cut bands with an intensity ratio of approximately 50/50. Since the mother’s blood film did not show a dimorphic distribution and the methylation study identified random X inactivation, the sideroblastic anaemia in this family was apparently not X linked. Interestingly, the DXS255 locus, detected by the M27β probe, maps to Xpl.12, just distal to the ALAS2 locus at Xpl.21.39 Although the recombination frequency between DXS255 and ALAS2 is not known, the different genotypes of M27β for case 1 versus 2 are consistent with inheritance of different ALAS2 alleles.

This is only the second case of pyridoxine-refractory congenital sideroblastic anaemia that is clearly autosomal recessive. The first case was the result of an autosomal pyridoxal kinase defect.40 However, this was apparently not the cause of sideroblastic anaemia in the present family as neither sib responded to pyridoxal 5’-phosphate therapy. Potentially, an autosomal gene involved in iron metabolism or in regulation of ALAS2 activity could contribute to the pathogenesis of sideroblastic anaemia in this family.

While there have been a few additional cases
with features consistent with autosomal inheritance. Amos et al. showed transmission from father to son and van Waveren Hogervorst et al. described a sideroblastic anaemia family with autosomal dominant inheritance of erythrocite dimorphism. Published reports of females with congenital sideroblastic anaemia who presented during infancy or early childhood, suggestive of autosomal inheritance, are listed in the table. The case reported by Sansone et al. described an affected girl of consanguineous parents in whom vacuolized marrow precursor cells were present. In Pearson syndrome the features of macrocytic anaemia, vacuolization of the bone marrow precursors, neutropenia, and thrombocytopenia are the result of mitochondrial DNA (mtDNA). Although there was normal endocrine pancreatic function, the case of Sansone et al. may represent a variant of Pearson syndrome. The involvement of a mtDNA deletion in the pathogenesis of the sideroblastic anaemia in the family reported here was unlikely as neither of the affected members had any of the clinical features associated with Pearson syndrome. Further molecular PCR analysis of mtDNA from the sibs provided no evidence for a mtDNA deletion (data not shown).

Notably, where evaluated, all of the cases listed in the table were pyridoxine-refractory. In contrast, many cases have been reported of XLSA which are pyridoxine-responsive. The first published mutation of the ALAS2 gene in a patient with XLSA was a defect in exon 9, the location of the putative pyridoxal 5'-phosphate binding site. This patient was pyridoxine-responsive, as was the mutant enzyme expressed in vitro. In addition, other pyridoxine-responsive patients with XLSA have recently been shown to have mutations in ALAS2. The family reported here, in addition to all of the presumed cases of autosomal inheritance of sideroblastic anaemia (table), were pyridoxine-refractory. Thus, we suggest that in cases of pyridoxine-refractory sideroblastic anaemia autosomal recessive mutations should be considered.

The family we have described has a uniquely severe, pyridoxine-refractory, trans-fusion dependent disorder that is not the result of a mutation in the ALAS2 gene. The exclusion of ALAS2 in the disease pathogenesis, the evidence against other forms of X-linked sideroblastic anaemia by methylation analysis, and the absence of any haematological manifestations in the parents strongly suggest that the sideroblastic anaemia in this family is caused by an autosomal recessive gene. Proof of this awaits identification of the mutation causing sideroblastic anaemia in this family. In addition, the availability of an informative ALAS2 polymorphism, which can be easily detected by mismatch PCR and XmnI digestion, and the recently described intron 7 polymorphism, will be useful in additional studies of ALAS2 and sideroblastic anaemia.

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Female children with congenital sideroblastic anaemia*

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*Values listed are for the earliest age at which the complete data were reported.
†Refractory to oral pyridoxal 5'-phosphate treatment only, pyridoxine responsiveness not reported.
¶Unresponsive to pyridoxine but responsive to pyridoxal 5'-phosphate.
∥Consanguineous parents.


26 Hendriks RW, Hinds H, Chen ZY, Craig IW. The hypervariable DXS255 locus contains a LINE-1 repetitive element with a CpG island that is extensively methylated only on the active X chromosome. Genomics 1992;14:598–603.


