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Familial-skewed X-chromosome inactivation as a predisposing factor for late-onset X-linked sideroblastic anemia in carrier females

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X-linked sideroblastic anemia (XLSA) is caused by mutations in the erythroid-specific 5-aminolevulinic acid synthase (*ALAS2*) gene. An elderly woman who presented with an acquired sideroblastic anemia is studied. Molecular analysis revealed that she was heterozygous for a missense mutation in the *ALAS2* gene, but she expressed only the mutated gene in reticulocytes. Her 2 daughters and a granddaughter were heterozygous for this mutation, had normal hemoglobin levels, and expressed the normal *ALAS2* gene in reticulocytes. A grandson with a previous diagnosis of thalassemia intermedia was found to be hemizygous for the *ALAS2* mutation. Treatment with pyridoxine completely corrected the anemia both in the proband and her grandson. All women who were analyzed in this family showed skewed X-chromosome inactivation in leukocytes, which indicated a hereditary condition associated with unbalanced lyonization. Because the preferentially active X chromosome carried the mutant *ALAS2* allele, acquired skewing in the elderly likely worsened the genetic condition and abolished the normal *ALAS2* allele expression in the proband. (Blood. 2000;96:4363-4365)

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Introduction

X-linked sideroblastic anemia (XLSA) is caused by mutations (primarily missense) in the erythroid-specific 5-aminolevulinic acid synthase (*ALAS2*) gene.[1] Affected hemizygous males may present in the first 2 decades of life with symptoms of anemia or in middle age with manifestations of secondary iron overload.[2] The majority of heterozygous females have no clinical signs because immature red blood cells (RBCs) expressing the normal *ALAS2* are sufficient to sustain a normal level of RBC production. As in any X-linked disorder, however, the clinical phenotype of female carriers may be influenced by the pattern of X-chromosome inactivation (or lyonization).[3] Different genetic mechanisms may lead to a skewed pattern of X-chromosome inactivation in females.[4,5] In addition, recent studies have shown that skewed lyonization can also be an acquired pattern in hematopoietic cells.[6-9]

Late-onset XLSA has been already described.[10] By studying the molecular basis for late-onset XLSA in an elderly woman, we report here a potential mechanism involving late-onset X-linked disorders in female obligate carriers of X-linked hematopoietic disorders.

Study design

The proband is a 72-year-old female of Italian ancestry. This woman was an active farm worker until her forties. At the age of 36 her hemoglobin (Hb) level was normal (Table 1). She later presented at the age of 64 with breathlessness and fatigue and was found to have severe microcytic anemia, with a Hb level of 52 g/L (5.2 g/dL; reference range, 120-160 g/L [12-16 g/dL] and a mean cell volume (MCV) of 74 fL (reference range, 83-97 fL). A presumptive diagnosis of myelodysplastic syndrome (refractory anemia with ringed sideroblasts) was made, and a transfusion therapy (3-4 units per month) was started.

When we saw the proband at the age of 71 years, her serum ferritin level was 3954 μg/L (female reference range, 12-200 μg/L) and transferrin saturation was 96% (female reference range, 15% to 45%), which indicated severe iron overload. She was treated with 4000 U/d subcutaneous (sc) recombinant human erythropoietin for 5 days a week and 300 mg/d oral pyridoxine. Four weeks later, her Hb level had increased from 77 to 176 g/L (7.7 to 17.6 g/dL); administration of erythropoietin was immediately discontinued, while oral pyridoxine was maintained. Hb levels stabilized between 110 and 120 g/L (11 and 12 g/dL) with no transfusion requirement, and MCV values ranged from 80-85 fL. Iron chelation therapy with sc deferoxamine was started.

Based on both RBC microcytosis and pyridoxine responsiveness, we hypothesized that this patient had late-onset X-linked sideroblastic anemia.[10] A careful review of family history revealed that an anemic grandson had been presumptively diagnosed with thalassemia intermedia. This 14-year-old boy was given pyridoxine and folic acid, although there was no evidence of folate deficiency, and his Hb level normalized, while MCV values remained low (Table 1). He was still following his daily course of 300 mg pyridoxine when we saw him at the age of 29.

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. Molecular analysis of the *ALAS2* gene was performed as previously described.[2] Analysis of X-chromosome inactivation was performed as previously described in detail, with minor modifications.[8] Relative expression of mutant and wild-type ALAS2 messenger RNAs (mRNAs) was evaluated in peripheral blood reticulocytes from the proband and her relatives.[11]

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Supported by a grant from MURST, Rome, Italy; a grant from the IRCCS Policlinico S. Matteo and a grant (M.C.) from the Fondazione Ferrata Storti, Pavia, Italy; grant R01 DK40895 (D.F.B.) from the National Institutes of Health, Bethesda, MD; and grant 584 (D.F.B.) from the March of Dimes Birth Defects Foundation.

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Table 1. Hematological data, ALAS2 exon 9 sequence (from 1230 to 1240 nucleotide), and results of X-chromosome inactivation studies using HUMARA assay in the family members

Subject	Age, y	Hb, g/L	MCV, fL	RDW, %	Serum ferritin, µg/L	ALAS2 paternal allele	ALAS2 paternal allele	HUMARA cleavage ratio* between alleles
Proband								
Before manifestations	36	136	ND	ND	ND			
At clinical onset	64	52	64	ND	ND			
Under transfusion	71	70	81	22.5	3954			
Under pyridoxine	74	123	85	18.9	2430	TTGGCTATGTG	TTGGCT**G**TGTG†	Not informative
Daughter II-2	52	137	88	17.7	110	TTGGCTATGTG	TTGGCT**G**TGTG†	3.2
Daughter II-3	50	132	92	17.1	126	TTGGCTATGTG	TTGGCT**G**TGTG†	4.0
Grandson III-1								
At diagnosis	14	79	63	ND	384			
Under pyridoxine	29	145	72	18.9	810	—	TTGGCT**G**TGTG	
During phlebotomy	30	138	78	16.1	122			
After phlebotomy	31	146	73	19.3	89			
Granddaughter III-2	32	130	85	16.6	82	TTGGCTATGTG	TTGGCT**G**TGTG	4.0
Normal range		F 12.0 -16.0 M 13.5-17.5	83-97	11-14	F 12 -200 M 20-250			

RDW indicates RBC distribution width, ie, the mean SD of RBC volume expressed as a percentage of the mean RBC volume. The ALAS2 paternal allele is a wild-type sequence: TTGGCTATGTG. Mutations are shown as bold fonts. ND indicates not done; F, female; M, male.

*If a woman is a perfect mosaic (50% of cells expressing the paternally derived X chromosome and 50% expressing the maternally derived one), the cleavage ratio is 1. A cleavage ratio of greater than 3 was previously established as the cut-off between cases with balanced X-chromosome inactivation (ratio, < 3) and cases with excessive skewing (ratio ≥ 3).[7]

†The mutated allele is supposed to be of maternal origin because the father did not have any manifestation of congenital anemia and therefore could not be a hemizygote.

Results and discussion

The pedigree of the proband's family is shown in Figure 1A. A single-point mutation in exon 9 of the *ALAS2* gene was found in 5 family members (Table 1). This is a transition from G to A at nucleotide 1236 that predicts an amino acid change of cysteine to tyrosine at position 395 (TGT→TAT; C395Y). Position 395 is very close to the lysine at position 391, which forms the Schiff base with the PLP aldehyde group. The point mutation might therefore interfere with binding of PLP or with the catalytic reaction itself. All 4 women studied, including the proband, were heterozygous for this exon 9 mutation (Table 1). The grandson had the mutation and therefore was a typical hemizygote. Studies on 200 normal alleles indicated that the transition from G to A at the 1236 nucleotide is not a polymorphism.

Analysis of DNA from peripheral blood leukocytes for polymorphisms at 2 X chromosome loci showed a status of homozygosity for all the loci examined: human androgen receptor (HUMARA), phosphoglycerate kinase (PGK), and DXS255 (probe M27β) (Figure 1B, HUMARA assay). This prevented clonal analysis of hematopoiesis to

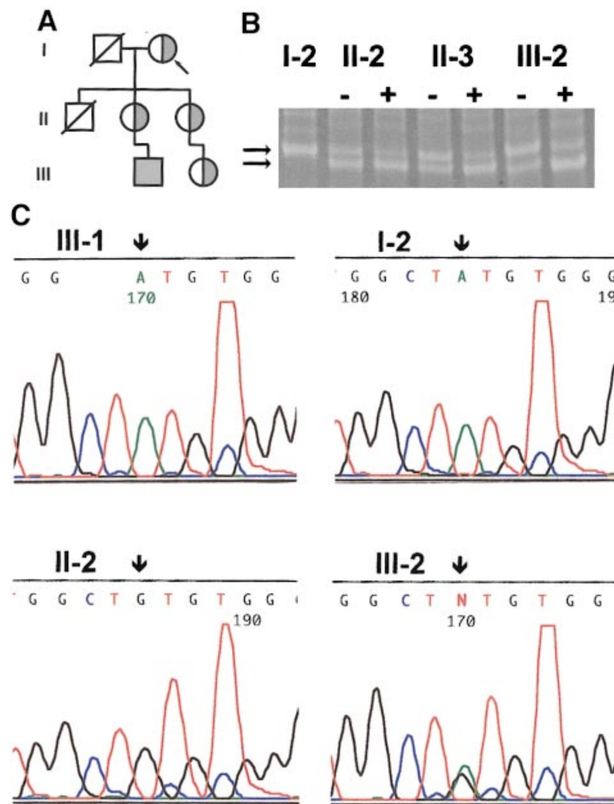

Figure 1. Molecular analysis of ALAS2 and clonal analysis of hematopoiesis. (A) Pedigree of the family. Circles denote female family members; squares, male family members; and symbols with diagonal lines, deceased members. The proband is denoted by an arrow. All women were heterozygotes for the *ALAS2* mutation; the only available male was hemizygote. (B) Clonal analysis of hematopoiesis using HUMARA assay on DNA from peripheral blood leukocytes. The − and + signs indicate sample aliquots undigested (−) or digested (+) with the methylation-sensitive restriction endonuclease *Hpa*II. *HUMARA* alleles (indicated by arrow) are represented by the lower band in the proband's sample (I-2, homozygous woman), and by the 2 lower bands in the other samples (heterozygous women). Upper bands probably derive from intrastrand secondary structures due to the high G + C content. In *Hpa*II digested samples from all the heterozygous women, the lower allele (paternally derived) is amplified more than the upper allele (maternally derived) compared with undigested samples. This indicates that the maternally derived *HUMARA* allele (carrying the mutant *ALAS2*) was less methylated (more active) and more digested by *Hpa*II than the paternally derived allele. In conclusion, unbalanced X-chromosome inactivation, leading to prevalent inactivation of the paternally derived chromosome (carrying the normal *ALAS2* allele), occurred in hematopoietic cells from these women. (C) Wild-type and mutant ALAS2 mRNA expression in reticulocytes from 4 family members. RNA was isolated from peripheral blood reticulocytes and reverse transcribed into cDNA; arrows indicate nucleotide 1236 of ALAS2 cDNA. III-1 (hemizygous grandson): As expected, only mutant cDNA (carrying A [adenine] at position 1236) was amplified. I-2 (proband): Only mutant cDNA was amplified, indicating that most of the reticulocyte ALAS2 mRNA derived from the mutant allele. II-2 (elder daughter): Only wild-type cDNA (carrying [guanine] at position 1236) was amplified, indicating that most of the reticulocyte ALAS2 mRNA derived from the wild-type allele. III-2 (granddaughter): Both wild-type and mutant cDNA were amplified. (N indicates the ambiguity resulting from overlapping signals corresponding to both guanine and adenine.)

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be carried out in the proband. The initial screening at the HUMARA locus revealed that the proband's daughters and granddaughter were heterozygous (Figure 1B). All 3 women had skewed X-chromosome inactivation because their cleavage ratios between alleles ranged from 3.2 to 4.0 (Table 1), and the preferentially active X chromosome carried the mutant *ALAS2* allele.

Sequence analysis of complementary DNA (cDNA) derived from reticulocyte RNA (Figure 1C) revealed that the proband and her grandson (both under pyridoxine treatment) expressed only the mutated *ALAS2* allele, with the G to A transition at nucleotide 1236. On the contrary, the elder heterozygous daughter expressed exclusively the wild-type allele, and the granddaughter expressed both the wild-type and mutated alleles. No female in the family exhibited the cytosine to guanine mutation in the XIST minimal promoter found by Plenge et al.[5] This does not exclude the possibility of a different mutation in the same gene involving XIST underexpression and preferential inactivation of the X chromosome carrying such mutation.

This case is interesting not only because the patient is a female and XLSA, as all the X-linked recessive diseases, normally affects hemizygous males, but also because the anemic condition was acquired in spite of the fact that the *ALAS2* defect was present from birth. In addition, skewed lyonization in leukocytes with preferential inactivation of the X chromosome carrying the normal *ALAS2* allele was found in all women examined, which clearly indicates that congenital skewing was also present in this family.[12] The available evidence suggests that late in this woman's life, an additional event following inheritance of the *ALAS2* mutation and congenital skewing led to dominance of hematopoietic cells expressing the X chromosome with the mutant gene.

The most likely explanation of the above findings is that the proband, despite a markedly unbalanced X-chromosome inactivation in her hematopoietic cells, was able to produce normal amounts of RBCs for the first 6 decades of her life, as do her daughters and granddaughter. In the seventh decade she developed acquired skewing, as do approximately one-third of elderly women.[13] She unfortunately further inactivated the parental X chromosome carrying the normal *ALAS2* gene, and when nearly all RBC precursors expressed the mutant gene, she became severely anemic.

A search of the literature reveals that at least 8 cases of late-onset pyridoxine-responsive sideroblastic anemia have been described (including the present case),[14-17] and 7 of these patients are women. Interestingly, Aivado et al[17] have recently described a family from Germany with a proven *ALAS2* mutation present in the mother and 2 of her 3 daughters. Excessively skewed lyonization was found in all women, and 2 of them developed microcytic anemia. Therefore, a combination of congenital and acquired skewing was likely present also in this family, although age-dependency was less clear.

Clinicians should be alerted to the possibility that an elderly woman may present with manifestations of X-linked hematopoietic disorders. In addition to XLSA, these may include glucose-6-phosphate dehydrogenase deficiency,[18] X-linked agammaglobulinemia or hyper-immunoglobulin (Ig) M (hyper-IgM) syndrome,[19] severe combined immunodeficiency,[20] and chronic granulomatous disease.[21,22]

Acknowledgments

We thank Joyce Hoy and Barrie Francis, Department of Medical Biochemistry, University of Wales College of Medicine, Cardiff, Wales, for their help with the ABI 377 electrophoresis and analysis.

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