RESEARCH ARTICLE

X-linked sideroblastic anemia due to ALAS2 intron 1 enhancer element GATA-binding site mutations

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X-linked sideroblastic anemia (XLSA) is the most common form of congenital sideroblastic anemia. In affected males, it is uniformly associated with partial loss-of-function missense mutations in the erythroid-specific heme biosynthesis protein 5-aminolevulinate synthase 2 (ALAS2). Here, we report five families with XLSA owing to mutations in a GATA transcription factor binding site located in a transcriptional enhancer element in intron 1 of the *ALAS2* gene. As such, this study defines a new class of mutations that should be evaluated in patients undergoing genetic testing for a suspected diagnosis of XLSA.
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Introduction

The sideroblastic anemias are a heterogeneous group of inherited and acquired hematological disorders characterized by the presence of ring sideroblasts—erythroblasts containing pathological mitochondrial iron deposits—in the bone marrow. The inherited or congenital sideroblastic anemias (CSAs) are uncommon diseases, typically characterized by germline genetic mutations leading to defects in mitochondrial heme synthesis, iron–sulfur cluster metabolism, or protein synthesis (reviewed in Ref. 1). Currently, nearly 60% of CSAs can be attributed to a mutation in a specific nuclear-encoded gene or mitochondrial DNA deletion [2]. Two-thirds or more of all genetically explained cases are due to mutations in 5-aminolevulate synthase 2 (*ALAS2*), the erythroid-specific isoform of the first, and rate-limiting, enzyme in heme biosynthesis, located on the *X* chromosome. Mutations in *ALAS2* lead to a hypochromic, microcytic CSA that occurs most commonly in males, and is commonly referred to as X-linked sideroblastic anemia (XLSA). All experimentally validated ALAS2 mutations described thus far in male probands are missense mutations, most often in domains important for catalysis or pyridoxal phosphate (vitamin B6) cofactor binding. In many cases, the anemia is responsive to high-dose dietary supplementation with pyridoxine, which stabilizes or otherwise promotes the activity of the mutant protein [3]. Females develop anemia in the setting of acquired unfavorably skewed *X* chromosome inactivation, and, unlike males, may have predicted null mutations that prematurely truncate the protein [4]. Clinically unaffected heterozygous carrier females may have a frankly bimodal red cell size distribution, but, more often, have subtle laboratory and morphological red blood cell (RBC) abnormalities such as an increased RBC distribution width (RDW) and occasional hypochromic microcytic RBCs or siderocytes on the peripheral blood smear. In some cases, they may have no morphological abnormalities whatsoever.

Additional Supporting Information may be found in the online version of this article.

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Methods

Probands in each family were ascertained based on the evaluation of clinical and pathological features, including a bone marrow aspiration showing ringed sideroblasts. Patient and family member samples for investigational studies were obtained with informed consent. Peripheral blood genomic DNA was used for all analyses. Except as noted, all genomic coordinates refer to the human genome sequence alignment release 19 (GRCh37/hg19) and were accessed using the UCSC Genome Browser (http://genome.ucsc.edu/) [5]. Genotyping was performed with Affymetrix 6.0 SNP chips in families A, F, and P using standard protocols. These three families are a subset of ~300 typed individuals in a collaborative CSA database (M.M.H., D.R.C., A.M., C.K., B.G., C.N., D.W.S., S.S.B., and M.D.F., unpublished) maintained at Boston Children's Hospital, and analyzed using a custom build, rule-based pipeline that integrates copy number variation (CNV) and family linkage with population level homozygosity and allele sharing. A detailed description of the software will be published elsewhere (Schmitz-Abe et al., unpublished). We delineated the minimal 17.3-Mb interval on chromosome *X* shared between the three American families (A, F, and P) using this pipeline and a 98% allelic identity threshold for pairs of individuals. In addition to CNV analysis based on Affymetrix 6.0 typing, we tested a subset of samples for small deletions using a custom Nimblgen 720k array covering chr*X*: 50,040,914–79,999,613 (hg18), with a median probe density of 50 bp. Data analysis was performed using Nimblescan 2.6. Bidirectional Sanger sequencing of patient genomic DNA was performed on samples amplified by polymerase chain reaction (PCR) using the primers listed in Supporting Information Table I. For quantitative real-time (RT)-PCR, total erythrocyte RNA was prepared from heparinized blood [6]. RNA was treated with DNase I (Roche) to remove contaminating genomic DNA. cDNA was synthesized using the iScript™ cDNA Synthesis Kit (Bio-Rad) according to the manufacturer's protocol. RT-PCR quantification of *ALAS2* [7] and α-globin-stabilizing protein (*AHSP*) [8] mRNA transcript levels was performed as described previously [9]. *X*-inactivation was performed using the HUMARA assay [10]. In Table I, in carrier females, the relative "activation" of the mutant allele compared to the wild-type allele is expressed as a fraction. A ratio of 1 indicates that there is equal activation of both alleles, whereas ratios of >1 indicate that the *X* chromosome carrying the mutant *ALAS2* is more active than the wild-type *X* chromosome (and vice versa).

Results and Discussion

We ascertained five families of Northern European descent—three American, one Dutch, and one British—in which affected male probands had hypochromic, microcytic CSA that was in several cases minimally or partially responsive to pyridoxine supplementation (Fig. 1A and Table I). In three pedigrees (Fig. 1A, Families A, F, and N), there was strong evidence of *X* linkage by the presence of multiple affected males distantly related through the maternal lineage and/or multiple generations of females, but not males, with hematological features consistent with XLSA carrier status. In one family, a female sibling (N-IV-3) of the three affected brothers was found to have iron overload and clinically significant hematologic abnormalities upon family screening. In this individual, the HUMARA *X*-inactivation assay was not informative; however, the degree of hematological abnormality, particularly the RDW, tended to be greater in those females in this and all other families with *X*-inactivation skewed toward the mutated *X* chromosome. Male probands did not have mutations in the exons and intron–exon boundaries of *ALAS2* or *GLRX5* (all families) or *SLC25A38* (families A, F, P, and N) [11,12]. In two families (Fig. 1A, Families A and F), deletions greater than ~250 bp were excluded using Affymetrix 6.0 SNP genotypes and a custom genomic tiling array spanning the entire *ALAS2* locus to the nearest flanking genes. Comparison of the SNP haplotypes on the *X* chromosome in the three American families (Families A, F, and P), however, demonstrated partially overlapping blocks of allelic identity in the vicinity of the *ALAS2* locus: the minimally shared region spanned a distance of 17.3 Mb (hg 18, Chr. *X*: 44,922,272–72,277,688), including the entire *ALAS2* locus, and segregated with the phenotype and presumptive female carrier state in the two families (Families A and F) in which multiple relatives were available. Portions of this haplotype were not present in 134 other males with CSA and their male family members, suggesting that this was not an unusually common haplotype and that these three families were ancestrally related. Although none of these individuals had cerebellar ataxia or other syndromic features, mutations in the mitochondrial ATP-binding cassette transporter ABCB7, responsible for XLSA with ataxia (XLSA/A) [13], and located 2 Mb distal to the minimally conserved haplotype, were also excluded by sequencing PCR-amplified exons. Similarly, Family N had no *ABCB7* mutations detected by sequencing.

These data strongly suggested that the phenotype could be attributed to a mutation closely linked to *ALAS2*, if not a regulatory or other noncoding or splicing mutation affecting *ALAS2* expression itself. To this end, in probands from all five families, we examined previously described and putative regulatory elements that promote erythroid *ALAS2* expression, including: ~2.6 kilobases (kb) of sequence immediately upstream of the transcriptional start site (TSS), containing the putative promoter [14], as well as two regions (chr*X*: 55,083,769–55,085,031 and chr*X*: 55,142,627–55,148,312) located ~25 and ~85 kb upstream of the TSS with extensive histone H3 lysine 27 acetylation (H3K27Ac) in K562 human erythroleukemia cells [15]. We also sequenced a previously defined enhancer element in intron 8 [16] in these five individuals, as well as 68 other genetically undefined CSA probands. In each case, analysis revealed nothing other than common variants or rare, unique variants that did not cluster or occur within evolutionarily conserved sequences. The complete sequence of *ALAS2* intron 1 in families A and F, however, which also has extensive H3K27Ac modification in K562 cells [15], revealed a A>G transition at chromosomal position *X*: 55054635 [ChrX(GRCh37/hg19):g.55054635A>G, NM_000032.4:c.−15–2187T>C] as shown in Fig. 1B. Analysis of this locus in the remaining three index families demonstrated, novel variants in adjacent nucleotides in Families C [Chr*X*(GRCh37/hg19):g.55054636 T>C; NM_000032.4:c.−15–2188A>G) and N (Chr*X*(GRCh37/hg19):g.55054634G>C; NM_000032.4:c.−15–2186C>G), and, as expected, the same g.55054635A>G variant in Family P. These variants all fall in the core GATA sequence element of a phylogenetically conserved GATA family transcription factor binding site (consensus sequence: [A/G]GATA[A/G]). Variants in this sequence were not present in 95 other unexplained CSA probands (including the 68 sequenced for intron 8 variants) having a wide variety of syndromic and nonsyndromic features [2] or in 69 whole genomes (K.S.-A. and K. M., unpublished).

The potential importance of such GATA-binding sites in hematological diseases is illustrated by the existence of mutations in GATA-binding sites in occasional patients with pyruvate kinase deficiency [17], as well as mutations in GATA1 and GATA2 themselves in familial erythroid, megakaryocytic, and hematopoietic syndromes [18–20]. Previous studies by Wang et al. demonstrated that this *particular ALAS2* intron 1 sequence element displays strong enhancer activity both in transiently transfected K562 cells as well as a stably targeted K562 reporter assay cell line induced to differentiate along the erythroid lineage [21]. When mutated, this sequence is unable to promote transcription of the reporter, confirming its functional significance. Furthermore, as assessed by chromatin immunoprecipitation, binding to this site in GATA1-ER cells is strongly induced by treatment with estradiol, which regulates translocation of GATA1 protein linked to the estrogen receptor regulatory domain in this cell line, promoting terminal differentiation and marked upregulation of *Alas2* gene expression. More recent, systematic chromatin immunoprecipitation experiments indicate that this element and adjacent sequences, are occupied by GATA1 and TAL1 proteins [15], whose colocalization is strongly associated with active erythroid promoter and enhancer elements [22]. Finally, in vitro selection of GATA-binding sites demonstrates a near *absolute* requirement for the first three nucleotides—G, A, and T—in the GATA-binding site core, each of which is mutated in at least one of the families we studied, for

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TABLE I. Hematological and Genetic Characteristics of Families with *ALAS2* Intron 1 GATA-binding Site Mutations

Individual	Sex	Age at evaluation	Affected status	Genotype ChrX (GRCh37):	Hemoglobin (g/dL)	MCV (fL)	RDW (%CV)[a]	X-activation ratio[b]	Blood smear	Pyridoxine response	Transfused	Iron overload	Comment
N-IV-2	F	52	No	g.55054634 G/C	13.2	93	15.8	0.8	Normal	NA	NA	No	
N-IV-3	F	55	Yes	g.55054634 G/C	13.1	112	22.3	NI	Few macrocytes	unknown	NA	Severe	Phlebotomized
N-IV-4	F	59	No	g.55054634 G/G	12.6	90	10.9	NI	Normal	NA	NA	No	
N-IV-5	M	63	No	g.55054634 C/Y	14.3	92	11.5	NA	Few poikilocytes	NA	NA	No	
N-IV-6	M	65	Yes	g.55054634 C/Y	12.4	80	15.8	NA	Few poikilocytes	Yes[c]	No	Mild	
N-IV-8	M	66	Yes	g.55054634 C/Y	9.8	84	21.4	NA	Few hypochromic microcytes and elliptocytes	minimal	Yes	Severe	HCC, Chelated
N-IV-9	M	67	Yes	g.55054634 C/Y	13.2	95	19.2	NA	Few microcytes, macrocytes, and stomatocytes	minimal	No	Mild	Phlebotomized
N-V-1	M	25	Yes	g.55054634 C/Y	9.7	74	26.2	NA	Few target cells and teardrop cells	minimal	Yes	Moderate	Chelated
N-V-2	F	35	No	g.55054634 G/C	12.2	84	16.9	3.3	Few macrocytes, microcytes, and poikilocytes	NA	NA	No	
A-III-1	M	16	Yes	ND	ND	ND	ND	NA	ND	Unknown	Unknown	Unknown	Died of anemia at 16 years
A-III-2	F	80	No	g.55054635 A/A	14.6	84	13.8	0.7[b]	Normal	NA	NA		
A-III-3	F	70s	No	g.55054635 A/G	13.4	88	14.2	0.3	Rare hypochromic microcytes	NA	NA		
A-III-4	F	73	No	g.55054635 A/G	13.0	83	18.0	3.4	Few hypochromic microcytes, rare siderocytes	NA	NA		
A-III-6	F	62	No	g.55054635 A/A	14.3	96	12.2	1.0[b]	Normal	NA	NA		
A-IV-1	M	21	Yes	ND	ND	ND	ND	NA	ND	Unknown	Yes	Severe	Died of anemia at 21 years
A-IV-3	F	44	No	g.55054635 A/G	12.8	86	16.0	NI	Few hypochromic microcytes and rare siderocytes	NA	NA		
A-V-1	F	28	No	g.55054635 A/G	13.9	83	13.6	0.5	Few hypochromic microcytes and rare siderocytes	NA	NA		
A-V-2	F	26	No	g.55054635 A/G	13.9	94	13.1	NI	Rare siderocytes	NA	NA		
A-V-3	M	18	Yes	g.55054635 G/Y	6.2	68	33.5	NA	Marked hypochromia, microcytosis, and anisopoikilocytosis	Minimal	No	Moderate	Chelated
F-I-1	F	93	No	g.55054635 A/G	10.0	85	20.4	2.5	Few hypochromic microcytes, slight anisopoikilocytosis, and rare siderocytes	Unknown	No		
F-II-1	F	68	No	g.55054635 A/G	11.1[d]	78	17.9	NI	Few hypochromic microcytes and rare siderocytes	Partial	No		
F-III-1	M	41	Yes	g.55054635 G/Y	8.6	67	33.6	NA	Marked hypochromia, microcytosis, and occasional siderocytes	No	Yes	Severe	Chelated
F-III-2	M	38	Yes	g.55054635 G/Y	8.8	84	31.0	NA	Marked anisopoikilocytosis and hypochromia, numerous burr cells, and occasional Howell–Jolly bodies	No	Yes	Severe	Splenectomy, died of pulmonary embolism
F-III-3	F		No	g.55054635 A/G	NML[c]	NML[c]	NML[c]	NI	ND	NA	NA		
C-I-1	F	67	No	ND	13.3	91.6	13.1	ND	Normal	NA	NA		
C-II-1	M	41	Yes	g.55054636 C/Y	13.0	79	17.7	NA	Few hypochromic microcytes and elliptocytes	Minimal	No	Moderate	Phlebotomized, HFE C282Y/+
C-II-2	M	35	Yes	g.55054636 C/Y	12.0	81	18.7	NA	Few hypochromic microcytes, elliptocytes, and siderocytes	Minimal	No	Moderate	Phlebotomized, HFE C282Y/+
P-II-1	M	39	?Yes	ND	ND	low[c]	ND	ND	ND	NA	No		
P-II-2	M	34	Yes	g.55054635 G/Y	8.0	77	19.2	NA	Moderate hypochromia	Minimal	No	Moderate	Phlebotomized

[a] RDW reference range = 10.5–13.5% CV,
[b] Expressed as ratio of *active* mutant:wild type X chromosomes in carrier females or two wild type alleles in non-carrier females.
[c] Quantitative data not available.
On pyridoxine supplementation. Baseline hemoglobin reported to be ~9 g/dL.
Abbreviations: NA, not applicable; ND, not determined; NML, normal; HCC, hepatocellular carcinoma; NI, locus not informative for HUMARA X-inactivation assay.

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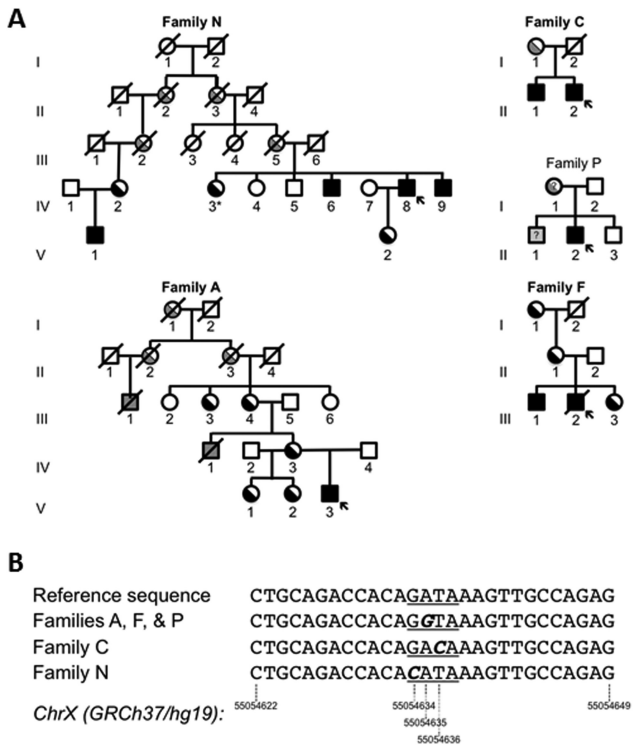

Figure 1. XLSA pedigrees with GATA-binding site mutations. A: Families A, F, and P are from the United States, N from The Netherlands, and C from Great Britain. The genotype or affected status of individuals studied is indicated by black shading. Individuals with inferred genotypes or phenotypes are shaded gray. An arrow indicates the proband in each family. Asterisk indicates a clinically affected heterozygous female. B: Sequence shown is on the positive strand of the GRCh37/hg19 reference sequence: Chr *X*:55,054,622–55,054,649. This corresponds to g.7849 to g.7876 in the *ALAS2* genomic sequence in the reverse orientation. The GATA-binding site is underlined. The mutation in each family is represented in bold italic font.

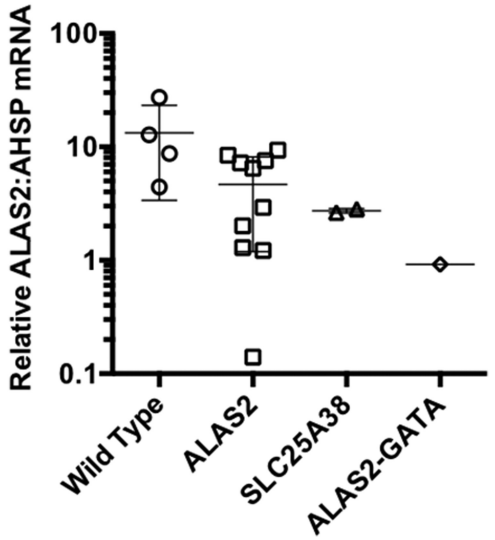

Figure 2. *ALASS* mRNA expression in GATA-binding site mutated patients. Peripheral blood erythrocyte *ALAS2* mRNA normalized to AHSP mRNA levels is shown in wild-type control and patients with *ALAS2* missense (ALAS2), *SLC25A38*, and *ALAS2-GATA*-biding site mutations. Whisker plots indicate mean ± one standard deviation. Data were qualitatively similar when *ALAS2* levels were normalized to *AHSP*, β-actin, or *SLC4A1* mRNAs.

binding to GATA1, GATA2, and GATA3 proteins [23,24]. Similarly, site-directed mutagenesis of other naturally occurring GATA-binding sites indicates that these three nucleotides are critical for complex formation, whereas the fourth nucleotide is less important. In fact, Plumb et al. compared the relative affinities of consensus GATA-binding elements to *precisely* the same nucleotide variants present in Families A, F, and P and C and found that these mutant oligonucleotides were <4,000- and <3,000-fold, respectively, less-efficient at competing the consensus probe in electrophoretic mobility shift assays (EMSAs) using mouse erythroleukemia cell nuclear extracts [25].

To determine if the mutations affected *ALAS2* mRNA abundance, we performed quantitative PCR using RNA isolated from patient and control peripheral blood RBC samples. As shown in Fig. 2, *ALAS2* mRNA levels normalized to the erythroid-specific *AHSP* showed that there was substantially less *ALAS2* mRNA in patient P-II-2 than in control individuals, patients with CSA owing to mutations in *SLC25A38*, and all but one XLSA patient with a missense mutation in *ALAS2*. Although limited to this single patient, the findings support the interpretation that mutations in this GATA element can result in decreased *ALAS2* mRNA expression.

Our findings strongly indicate that the conserved GATA element is an erythroid enhancer and that the patient-associated alleles are functionally significant mutations owing to the interruption of GATA1 binding. It is not possible to make definitive genotype–phenotype correlations, as even within a family (e.g., Family N) there is a great deal of phenotypic variability, suggesting a variety of modifying factors, both genetic and otherwise.

During the course of this study, we became aware of a similar study by Kaneko et al. [26], who confirmed the presence of a functional GATA-binding site-dependent erythroid enhancer element in intron 1 of *ALAS2*, using an approach entirely independent of that published previously [21]. Therein, they also describe mutations in the enhancer in three Japanese male probands with hypochromic, microcytic CSA; one of these was from an extended pedigree, demonstrating apparent *X*-linkage. One mutation, 55054635A>G, present in two of the probands is identical to that found in Families A, F, and P and the other is a 37-bp deletion that encompasses the entire GATA-binding site. They confirm that the former mutation, in concordance with the findings of Wang et al. [21], as well as the novel deletion, abrogates DNA binding in EMSAs and that both mutations substantially diminish erythroid-specific enhancer activity. Finally, similar to our observation, they show a decrease in ALAS2 expression in primary cells from one patient.

Conclusions

Taken together, these genetic and functional data strongly support the conclusion that mutations in an enhancer element in *ALAS2* intron 1 that contains a GATA-binding site result in a clinical phenotype similar to patients with XLSA owing to mutations in the *ALAS2* coding sequence itself. In our own survey of >125 probands with CSA (Ref. 2 and data not shown), *ALAS2* intron 1 enhancer element mutations constitute ~3% of all probands and ~5% of cases of XLSA. For this reason, we suggest that *ALAS2* intron 1 GATA-binding site variants can be excluded in males with hypochromic, microcytic, nonsyndromic sideroblastic anemias lacking coding mutations in *ALAS2* or biallelic coding or splicing variants in *SLC25A38*, particularly those with clinical or laboratory evidence of *X*-linkage or a minimal, but discernable, response to pyridoxine therapy.

Author Contributions

M.D.F. supervised all aspects of the project, including analysis of Families A, F, and P, designed genomic SNP, CNV, and sequence

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analyses and wrote the manuscript. D.R.C. designed, performed, analyzed, and annotated sequence analysis on Families A, F, and P and designed, performed, analyzed most of the supporting sequence data in the coding regions of ALAS2, SLC25A38, ABCB7, and GLRX5 in probands in the Boston Children's Hospital cohort. K.S.-A. designed and constructed SNP pipeline (Variant Explorer) and performed SNP and CNV analyses. P.J.S. performed quantitative PCR analysis of *ALAS2* mRNA. M.M.H. coordinated human subject research protocols, CSA sample collection, and database management at Boston Children's Hospital. A.S. performed X-inactivation studies in Families A and F. K.S.-A. analyzed SNP, CNV, and sequence analyses. K.M. supervised SNP and CNV analyses and edited the manuscript. S.S.B. ascertained, clinically phenotyped families A, F, and P, and edited the manuscript. D.W.S. coordinated and supervised sequence and phenotypic analysis of Family N and edited the manuscript. R.v.W. supervised phenotypic analysis of Family N and edited the manuscript. C.I.d.B., R.A.R., and N.V.A.M.K. ascertained and clinically phenotyped Family N. H.I.J. performed X-chromosome inactivation assays on Family N. A.M. and M.S. performed sequence and hematological phenotypic analyses of Family C and edited the manuscript. G.M. and S.S. ascertained and clinically phenotyped Family C. C.K. phenotyped patients and contributed DNA samples for genetic analysis and edited the manuscript. B.G. and C.M.N. phenotyped patients and contributed DNA samples for genetic analysis.

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