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.


Introduction

The sideroblastic anemias are a heterogeneous group of inherited and acquired hematological disorders characterized by the presence of ring sideroblasts—erythroid blasts 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-aminolevulinate 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.

Conflict of interest: Nothing to report.
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Proband(s) 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 investigative 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 assembly GRCh37/hg19. CGH analysis was performed using Agilent 4×44K whole-genome CGH arrays (Agilent Technologies). We delineated the minimal 17.3-Mb interval on chromosome X spanning a distance of 17.3 Mb (hg 18, Chr. X 72,277,688–72,450,320) including the entire ALAS2 gene and a 250-kb region flanking the gene on each chromosome arm (and vice versa).

## Methods

Proband(s) 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 investigative 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 assembly GRCh37/hg19. CGH analysis was performed using Agilent 4×44K whole-genome CGH arrays (Agilent Technologies). We delineated the minimal 17.3-Mb interval on chromosome X spanning a distance of 17.3 Mb (hg 18, Chr. X 72,277,688–72,450,320) including the entire ALAS2 gene and a 250-kb region flanking the gene on each chromosome arm (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 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 Nimblegen 720k array covering chrX: 50,040,914–79,999,613 (hg18), with a median probe density of 50 bp. Data analysis was performed using Nimblescan 2.6. Directed 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 (HSPB) [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).
<table>
<thead>
<tr>
<th>Individual</th>
<th>Sex</th>
<th>Age at evaluation</th>
<th>Affected status</th>
<th>Genotype Chr X (GRCh37):</th>
<th>Hemoglobin (g/dL)</th>
<th>MCV (fL)</th>
<th>RDW (%CV)a</th>
<th>X-activation ratiob</th>
<th>Blood smear</th>
<th>Pyridoxine response</th>
<th>Transfused</th>
<th>Iron overload</th>
<th>Comment</th>
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<tbody>
<tr>
<td>N-IV-2</td>
<td>F</td>
<td>52</td>
<td>No</td>
<td>g.55054634 G/C</td>
<td>13.2</td>
<td>93</td>
<td>15.8</td>
<td>0.8</td>
<td>Normal</td>
<td>NA</td>
<td>NA</td>
<td>No</td>
<td>Phlebotomized</td>
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<tr>
<td>N-IV-3</td>
<td>F</td>
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<td>Yes</td>
<td>g.55054634 G/C</td>
<td>13.1</td>
<td>112</td>
<td>22.3</td>
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<td>Few macrocytes</td>
<td>NA</td>
<td>No</td>
<td>No</td>
<td>Splenectomy</td>
</tr>
<tr>
<td>N-IV-4</td>
<td>F</td>
<td>59</td>
<td>No</td>
<td>g.55054634 G/G</td>
<td>12.6</td>
<td>90</td>
<td>10.9</td>
<td>Normal</td>
<td>Normal</td>
<td>NA</td>
<td>NA</td>
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<td>Splenectomy</td>
</tr>
<tr>
<td>N-IV-5</td>
<td>M</td>
<td>63</td>
<td>No</td>
<td>g.55054634 C/Y</td>
<td>14.3</td>
<td>92</td>
<td>11.5</td>
<td>NA</td>
<td>Few poikilocytes</td>
<td>NA</td>
<td>No</td>
<td>No</td>
<td>Severe</td>
</tr>
<tr>
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<td>65</td>
<td>Yes</td>
<td>g.55054634 C/Y</td>
<td>12.4</td>
<td>80</td>
<td>15.8</td>
<td>NA</td>
<td>Few poikilocytes</td>
<td>NA</td>
<td>No</td>
<td>Mild</td>
<td>Splenectomy</td>
</tr>
<tr>
<td>N-IV-7</td>
<td>M</td>
<td>66</td>
<td>Yes</td>
<td>g.55054634 C/Y</td>
<td>9.8</td>
<td>84</td>
<td>21.4</td>
<td>NA</td>
<td>Few hypochromic microcytes and elliptocytes</td>
<td>minimal</td>
<td>Yes</td>
<td>Severe</td>
<td></td>
</tr>
<tr>
<td>N-IV-8</td>
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<td>67</td>
<td>Yes</td>
<td>g.55054634 C/Y</td>
<td>13.2</td>
<td>95</td>
<td>19.2</td>
<td>NA</td>
<td>Few microcytes, macrocytes, and stomatocytes</td>
<td>minimal</td>
<td>No</td>
<td>Mild</td>
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<tr>
<td>N-IV-9</td>
<td>M</td>
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<td>Yes</td>
<td>g.55054634 C/Y</td>
<td>9.7</td>
<td>74</td>
<td>26.2</td>
<td>NA</td>
<td>Few target cells and teardrop cells</td>
<td>minimal</td>
<td>Yes</td>
<td>Moderate</td>
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<tr>
<td>N-V-2</td>
<td>F</td>
<td>35</td>
<td>No</td>
<td>g.55054634 G/C</td>
<td>12.2</td>
<td>84</td>
<td>16.9</td>
<td>3.3</td>
<td>Few macrocytes, microcytes, and poikilocytes</td>
<td>NA</td>
<td>NA</td>
<td>No</td>
<td></td>
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<tr>
<td>A-III-1</td>
<td>M</td>
<td>16</td>
<td>Yes</td>
<td>g.55054634 C/Y</td>
<td>14.3</td>
<td>96</td>
<td>12.2</td>
<td>1.0</td>
<td>Normal</td>
<td>NA</td>
<td>NA</td>
<td>Severe</td>
<td></td>
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<tr>
<td>A-IV-1</td>
<td>M</td>
<td>21</td>
<td>Yes</td>
<td>g.55054634 C/Y</td>
<td>13.0</td>
<td>83</td>
<td>18.0</td>
<td>3.4</td>
<td>Few hypochromic microcytes, rare siderocytes</td>
<td>NA</td>
<td>NA</td>
<td>Moderate Chelated</td>
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<td>A-III-2</td>
<td>M</td>
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<td>No</td>
<td>g.55054635 A/G</td>
<td>14.6</td>
<td>84</td>
<td>13.8</td>
<td>0.7</td>
<td>Normal</td>
<td>NA</td>
<td>NA</td>
<td>Severe</td>
<td></td>
</tr>
<tr>
<td>A-III-3</td>
<td>M</td>
<td>18</td>
<td>Yes</td>
<td>g.55054635 G/Y</td>
<td>8.6</td>
<td>67</td>
<td>33.6</td>
<td>NA</td>
<td>Marked hypochromia, microcytosis, and anisopoikilocytosis</td>
<td>Minimal</td>
<td>No</td>
<td>Moderate</td>
<td></td>
</tr>
<tr>
<td>F-III-1</td>
<td>F</td>
<td>93</td>
<td>No</td>
<td>g.55054635 A/G</td>
<td>10.0</td>
<td>85</td>
<td>20.4</td>
<td>2.5</td>
<td>Few hypochromic microcytes, slight anisopoikilocytosis and rare siderocytes</td>
<td>Unknown</td>
<td>No</td>
<td>Splenectomy, died of pulmonary embolism</td>
<td></td>
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<tr>
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<td>M</td>
<td>41</td>
<td>Yes</td>
<td>g.55054635 G/Y</td>
<td>8.6</td>
<td>67</td>
<td>33.6</td>
<td>NA</td>
<td>Marked hypochromia, microcytosis, and occasional siderocytes</td>
<td>No</td>
<td>Yes</td>
<td>Severe</td>
<td></td>
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<tr>
<td>F-III-3</td>
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<td>g.55054635 G/Y</td>
<td>8.8</td>
<td>84</td>
<td>31.0</td>
<td>NA</td>
<td>Marked anisopoikilocytosis and hypochromism, numerous burr cells, and occasional Howell-Jolly bodies</td>
<td>No</td>
<td>Yes</td>
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<td>F-III-4</td>
<td>M</td>
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<td>g.55054635 A/G</td>
<td>11.1</td>
<td>78</td>
<td>17.9</td>
<td>NA</td>
<td>Few hypochromic microcytes and rare siderocytes</td>
<td>Partial</td>
<td>No</td>
<td>Splenectomy, died of pulmonary embolism</td>
<td></td>
</tr>
<tr>
<td>C-I-1</td>
<td>F</td>
<td>67</td>
<td>No</td>
<td>g.55054636 C/Y</td>
<td>13.3</td>
<td>91.6</td>
<td>13.1</td>
<td>ND</td>
<td>Normal</td>
<td>NA</td>
<td>NA</td>
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</tr>
<tr>
<td>C-II-1</td>
<td>M</td>
<td>41</td>
<td>Yes</td>
<td>g.55054636 C/Y</td>
<td>13.0</td>
<td>79</td>
<td>17.7</td>
<td>ND</td>
<td>Few hypochromic microcytes and elliptocytes</td>
<td>Minimal</td>
<td>No</td>
<td>Moderate</td>
<td></td>
</tr>
<tr>
<td>C-II-2</td>
<td>M</td>
<td>35</td>
<td>Yes</td>
<td>g.55054636 C/Y</td>
<td>12.0</td>
<td>81</td>
<td>18.7</td>
<td>NA</td>
<td>Few hypochromic microcytes, elliptocytes, and siderocytes</td>
<td>Minimal</td>
<td>No</td>
<td>Moderate Phlebotomized</td>
<td></td>
</tr>
<tr>
<td>P-I-1</td>
<td>M</td>
<td>39</td>
<td>Yes</td>
<td>g.55054635 G/Y</td>
<td>8.0</td>
<td>77</td>
<td>19.2</td>
<td>NA</td>
<td>Moderate hypochromia</td>
<td>Minimal</td>
<td>No</td>
<td>Moderate</td>
<td></td>
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<tr>
<td>P-I-2</td>
<td>M</td>
<td>34</td>
<td>Yes</td>
<td>g.55054635 G/Y</td>
<td>8.0</td>
<td>77</td>
<td>19.2</td>
<td>NA</td>
<td>Moderate hypochromia</td>
<td>Minimal</td>
<td>No</td>
<td>Moderate Phlebotomized</td>
<td></td>
</tr>
</tbody>
</table>

a RDW reference range 5 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.
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.

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-I-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.

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-binding 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.

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
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.

Acknowledgments

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References