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

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AM and MS performed sequence and hematological phenotypic analyses of Family C and edited the manuscript.
GM and SS ascertained and clinically phenotyped Family C.
CK phenotyped patients and contributed DNA samples for genetic analysis and edited the manuscript.
BG and CMN phenotyped patients and contributed DNA samples for genetic analysis.

Abstract

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 due 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—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 reference (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, enzymein 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) co-factor 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.

Methods

Probands in each family were ascertained based on 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 (MMH, DRC, AM, CK, BG, CN, DWS, SSB, and MDF, 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, & 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 chrX: 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 PCR using the primers described in Supplementary Table 1. For quantitative 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. Real-time PCR quantification of *ALAS2* (7) and *AHSP* (8) mRNA transcript levels were performed as described previously (9). X-inactivation was performed using the HUMARA assay (10). In Table 1, 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 >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 (Figure 1A and Table 1). In three pedigrees (Figure 1A, A, F, & 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 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 intronexon boundaries of *ALAS2* or *GLRX5* (all families) or *SLC25A38* (families A, F, P, and N) (11, 12). In two families (Figure 1A, Families A & F), deletions greater than ∼250 base pairs 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 (A, F, & 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 (A & 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 X-linked sideroblastic anemia with ataxia (XLSA/A) (13), and located 2 Mb distal to the minimally conserved haplotype, were also excluded by sequencing PCR-amplified exons. Likewise, 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 non-coding 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

(chrX:55,083,769-55,085,031 and chrX: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 Figure 1B. Analysis of this locus in the remaining three index families demonstrated, novel variants in adjacent nucleotides in Families C [ChrX(GRCh37/hg19):g.55054636 T>C; NM_000032.4:c.-15-2188A>G) and N (ChrX(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 non-syndromic features (2) or in 69 whole genomes (KS-A and KM, unpublished).

The potential importance of such GATA binding sites in hematological diseases is illustrated by the existence 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 up-regulation 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 co-localization is strongly associated with active erythroid promoter and enhancer elements (22). Lastly, *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 binding to GATA1, GATA2, and GATA3 proteins (23, 24). Site-directed mutagenesis of other naturally occurring GATA binding sites, likewise 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 <4000 and <3000-fold, respectively, less-efficient at competing the consensus probe in electrophoretic mobility shift assays (EMSAs) using mouse erythroleukemia (MEL) cell nuclear extracts (25).

In order 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 Figure 2, *ALAS2* mRNA levels normalized to the erythroid-specific α-globin stabilizing protein, *AHSP*, showed that there was substantially less *ALAS2* mRNA in patient P-II-2 than in control individuals, patients with CSA due 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 due to 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 work, 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 previously published (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, 55054635 A>G, present in two of the probands is identical to that found in families A, F, and P and the other is a 37 base pair 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. Lastly, similar to our observation, they show a decrease in ALAS2 expression in primary cells from one patient.

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 due to mutations in the *ALAS2* coding sequence itself. In our own survey of >125 probands with CSA (reference (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 be excluded in males with hypochromic, microcytic, non-syndromic 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.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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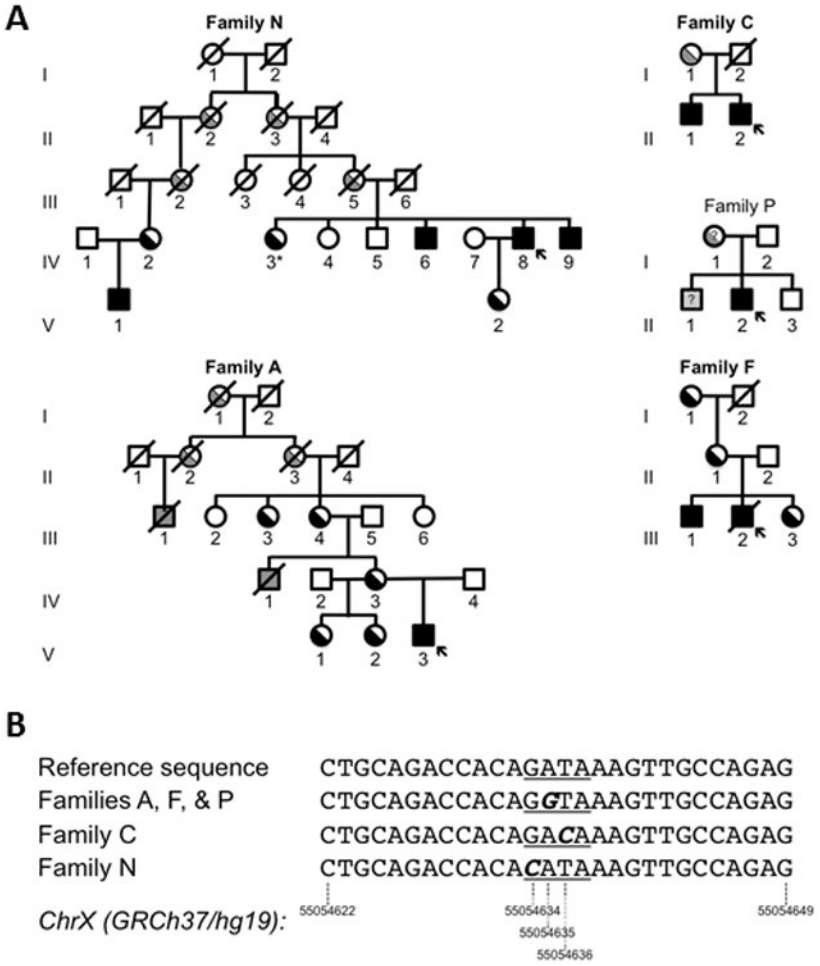

Figure 1. XLSA pedigrees with GATA binding site mutations

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 are indicated by black shading. Individuals with inferred genotypes or phenotypes are shaded gray. An arrow indicates the proband in each family. *Indicates a clinically affected heterozygous female.

B. Sequence shown is on the positive strand of the GRCh37/hg19 reference sequence: Chr X:55054622-55054649. 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 in bold italics.

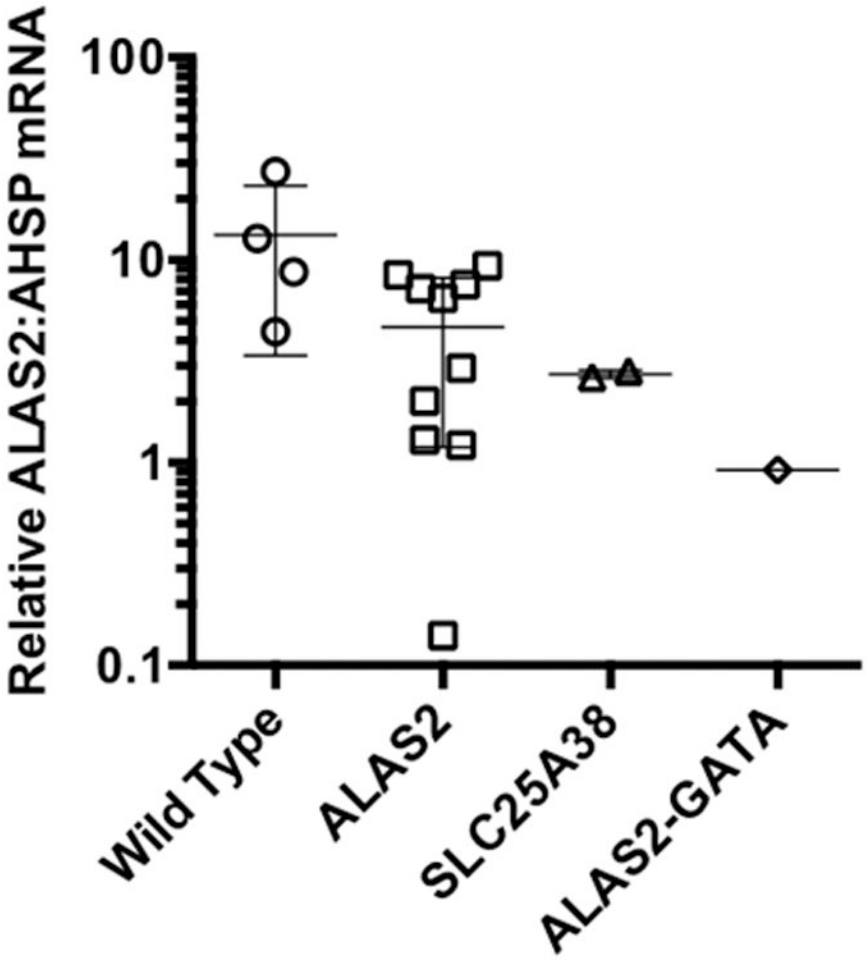

Figure 2. *ALASS* mRNA expression in GATA binding site mutated patients

Peripheral blood erythrocyte *ALAS2* mRNA normalized to alpha hemoglogin stabilizing protein (AHSP) mRNA levels are shown in wild type control and patients with *ALAS2* missense (ALAS2), *SLC25A38*, and *ALAS2-GATA* biding site mutations. Whisker plots indicate the mean ± one standard deviation. Data were qualitatively similar when *ALAS2* levels were normalized to *AHSP*, β-actin, or *SLC4A1* mRNAs.

Table 1

Hematological and genetic characteristics of families with *ALAS2* intron 1 GATA binding site mutations.

Individual	Sex	Age at evaluation	Affected Status	Genotype ChrX (GRCh37):	Hb (g/dl)	MCV (fl)	RDW (%CV)¶	X-activation ratio*	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&	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, poikilocytes	NA	NA	No	
A-III-1	M	16	Yes	ND	ND	ND	ND	NA	ND	unknown	unknown	unknown	Died of anemia age 16
A-III-2	F	80	No	g.55054635 A/A	14.6	84	13.8	0.7*	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*	normal	NA	NA		
A-IV-1	M	21	Yes	ND	ND	ND	ND	NA	ND	unknown	Yes	Severe	Died of anemia age 21
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†	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, 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&	NML&	NML&	NI	ND	NA	NA		

Individual	Sex	Age at evaluation	Affected Status	Genotype ChrX (GRCh37):	Hb (g/dl)	MCV (fl)	RDW (%CV)¶	X-activation ratio*	Blood smear	Pyridoxine response	Transfused	Iron Overload	Comment
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&	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

NA= not applicable, ND = not determined, NML=normal, HCC=hepatocellular carcinoma, NI=locus not informative for HUMARA X-inactivation assay.

¶RDW reference range = 10.5-13.5% CV,

*Expressed as ratio of *active* mutant:wild type X chromosomes in carrier females or two wild type alleles in non-carrier females.

&Quantitative data not available,

†On pyridoxine supplementation. Baseline Hb reported to be ~9 g/dl.