Table 4 Congenital sideroblastic anemia (other than XLSA)

Case number	Age at diag (y.o.)	Gender	Family history	Gene mutation							Hb (g/dl)	MCV (fl)	Response to Vit.B6
				ALAS2	*SLC25A38*	*GLRX5*	*ABCB7*	*SLC19A2*	*PUS1*	*SF3B1*			
11	19	M	−	−	−	−	−	−	−	−	7.8	73.9	−
12	4	M	−	−	−	−	−	−	−	−	6.6	73.6	−
13	0	M	+	−	−	−	−	−	−	−	3.9	65.0	−
14	20	M	+	−	−	−	−	−	−	−	7.6	82.0	+
15	0	M	−	N/D	N/D	N/D	N/D	N/D	N/D	N/D	6.8	88.1	N/D[a]
16	32	M	−	N/D	N/D	N/D	N/D	N/D	N/D	N/D	11.2	69	+
17	36	M	−	N/D	N/D	N/D	N/D	N/D	N/D	N/D	10.8	67.3	+
18	18	F	+	N/D	N/D	N/D	N/D	N/D	N/D	N/D	9.3	96.2	+

N/D not done

[a] Vit.B6 was not administered due to PMPS

+8 appeared to be more common in RCMD. In addition, −7 was identified in four patients with RCMD (14.8 %), whereas it was not identified in RARS. This difference may be related to the poor prognosis of RCMD.

Regarding the responsiveness to pyridoxine treatment among XLSA, 6 of 10 cases responded to Vit.B6 treatment in this study, although the magnitude of response varied among individuals. Thus, as the benefit of treatment of Vit.B6 for XLSA is obvious, a precise diagnosis of XLSA is important. As late-onset XLSA cases have been reported and two patients over 60 years old were found in this study, genetic analysis in sideroblastic anemia patients with microcytic anemia is essential regardless of age.

Focusing on *ALAS2* mutation in XLSA, two patients with the same mutation (c.509G>T), which results in R170L, showed distinct responses to Vit.B6. Edgar et al. [22] reported a Vit.B6 responsive pedigree with XLSA carrying the p.R170L mutation of ALAS2 gene. Furthermore, the crystal structure analysis of ALAS from *Rhodobacter capsulatus* [23] suggests that a missense mutation at Arg170 destabilizes PLP binding, which might be partially restored with excess amounts of PLP. Together with the findings of biochemical analysis in this study, it is strongly suggested that R170L mutation causes pyridoxine-responsive XLSA. However, in consistent with the data of in vitro analysis and clinical course of other R170L patients, case 10 was unresponsive to Vit.B6 treatment. Thus, onset and severity of the disease may be defined by not only the type of mutation but also the environmental and physiological status of the patients. This speculation may be supported by the results that there is a discrepancy between in vitro and in vivo response to Vit.B6 in some cases (Table 3).

The high incidence of XLSA among CSA in the present study was consistent with a previous report in the USA. Bergmann et al. [24] reported genetic analysis of CSA in the USA. In this study, mutations of *ALAS2*, *SLC25A38*, mitochondria DNA, and *PUS1*, were identified in 37, 15, 2.5, and 2.5 % of CSA cases, respectively. The most significant difference from our study was that mutations of the *SLC25A38* gene were frequently found in the USA. Since *SLC25A38* is thought to be a transporter of glycine, which is a substrate for ALAS2 in the first step of heme synthesis, the

Table 5 Mutation of *SF3B1* gene in MDS-RS

Case number	Diagnosis	Age at diagnosis (y.o.)	Gender	Chromosome anomaly	position of *SF3B1* mutation
1	RARS	82	M	−	E622D
2	RARS	57	M	−	N626S
3	RARS	60	M	Complex karyotype, including +8	K700E
4	RARS	60	M	−	K700E
5	RARS	73	F	−	No mutation
6	RARS	74	F	−	H662Q
7	RARS	76	M	−	K700E
8	RARS	67	F	−	K700E
9	RARS	66	M	−	K666E
10	RCMD	50	F	−	No mutation

(−) normal karyotype

pathology of CSA due to mutation of this gene is similar to that of XLSA. Therefore, CSA patients with microcytic anemia, in whom mutations of *ALAS2* gene were not identified, were expected to harbor *SLC25A38* mutation; however, it was not detectable in this study. To date, it has not been reported in Asia, although mutation of the *SLC25A38* gene has been widely reported in the USA, Canada, and Europe. Together with the results of the present study, it is suggested that the causative genes of CSA differ among races and regions.

Recently, mutations of genes involved in splicing machinery were reported in MDS [6]. Among them, *SF3B1*, which is a component of the U2-small nuclear ribonucleoprotein (U2-snRNP) complex [25], was found to be highly mutated in MDS with ring sideroblasts [6]. In this study, *SF3B1* mutation was examined in nine cases of CSA; however, its mutation was not detectable in CSA. These findings suggest that the mechanism for sideroblasts formation may be different between CSA and MDS.

In conclusion, our data showed that XLSA is the most frequent type of CSA; however, onset and severity of the disease may be affected by the environmental and physiological status of the patients. The data, including clinical and genetic analysis, further suggest that genetic background is different between CSA and MDS.

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Conflict of interest disclosure The authors declare no competing financial interest.

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Identification of a novel erythroid-specific enhancer for the *ALAS2* gene and its loss-of-function mutation which is associated with congenital sideroblastic anemia

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ABSTRACT

Erythroid-specific 5-aminolevulinate synthase (ALAS2) is the rate-limiting enzyme for heme biosynthesis in erythroid cells, and a missense mutation of the *ALAS2* gene is associated with congenital sideroblastic anemia. However, the gene responsible for this form of anemia remains unclear in about 40% of patients. Here, we identify a novel erythroid-specific enhancer of 130 base pairs in the first intron of the *ALAS2* gene. The newly identified enhancer contains a *cis*-acting element that is bound by the erythroid-specific transcription factor GATA1, as confirmed by chromatin immunoprecipitation analysis *in vivo* and by electrophoretic mobility shift assay *in vitro*. A promoter activity assay in K562 human erythroleukemia cells revealed that the presence of this 130-base pair region increased the promoter activity of the *ALAS2* gene by 10-15-fold. Importantly, two mutations, each of which disrupts the GATA-binding site in the enhancer, were identified in unrelated male patients with congenital sideroblastic anemia, and the lower expression level of ALAS2 mRNA in bone marrow erythroblasts was confirmed in one of these patients. Moreover, GATA1 failed to bind to each mutant sequence at the GATA-binding site, and each mutation abolished the enhancer function on ALAS2 promoter activity in K562 cells. Thus, a mutation at the GATA-binding site in this enhancer may cause congenital sideroblastic anemia. These results suggest that the newly identified intronic enhancer is essential for the expression of the *ALAS2* gene in erythroid cells. We propose that the 130-base pair enhancer region located in the first intron of the *ALAS2* gene should be examined in patients with congenital sideroblastic anemia in whom the gene responsible is unknown.

Introduction

The *ALAS2* gene encodes for erythroid-specific 5-aminolevulinate synthase (ALAS-E, EC 2.3.1.37), which is the rate-limiting enzyme of the heme biosynthetic pathway in erythroid cells.[1] It has been reported that the human *ALAS2* gene is mapped on the X chromosome,[2] and that a loss-of-function mutation of this gene causes X-linked sideroblastic anemia (XLSA),[3,4] which is the most common genetic form of congenital sideroblastic anemia (CSA). Moreover, a missense mutation of *ALAS2* was identified in a patient with non-familial CSA (nfCSA),[5] in which no family history of sideroblastic anemia was identified. In addition to *ALAS2*, several other genes were recently identified as causative genes for CSA, including *SLC25A38*,[6] *GLRX5*,[7] *ABCB7*,[8] *PUS1*,[9] and *SLC19A2*,[10] but the cause of sideroblastic anemia still remains undefined in more than 40% of patients with CSA.[11]

GATA1 transcription factor regulates the expression of several erythroid–specific genes, such as erythropoietin receptor gene,[12,13] α- and β-globin genes,[14,15] *ALAS2*[16] and the *GATA1* gene itself,[17] during erythroid differentiation.[18,19] Ablation of the *Gata1* gene in mice resulted in embryonic death because of anemia,[20] suggesting that GATA1 is essential for erythroid differentiation *in vivo*. It has been reported that GATA1 regulates transcription of human *ALAS2* through the proximal promoter region[16] and the erythroid-specific enhancer located in the eighth intron of *ALAS2*.[21] However, Fujiwara *et al.* demonstrated that the GATA1 protein binds to the *ALAS2* gene only in the middle of its first intron, where no regulatory region had so far been identified, by genome-wide analysis of K562 human erythroleukemia cells using chromatin immunoprecipitation followed by next-generation sequencing (ChIP-seq).[22]

In the present study, we have identified a novel erythroid-specific enhancer region in the first intron of the *ALAS2* gene. Moreover, we describe two mutations in the newly identified enhancer of *ALAS2*: a T-to-C transition, which changes GATA to GGTA at the GATA element in the antisense strand, in a pedigree with XLSA and one proband with nfCSA, and a 35-base pair (bp) deletion including the above-mentioned GATA element in a proband with nfCSA.

Methods

Polymerase chain reaction

DNA polymerases used for polymerase chain reaction (PCR) analysis were purchased from TAKARA BIO Inc. (Shiga, Japan). The

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sequence of primers and probes used in this study are listed in the *Online Supplementary Tables*.

Polymerase chain reaction-based quantitative chromatin immunoprecipitation

Real-time PCR-based quantitative chromatin immunoprecipitation (ChIP-qPCR) analysis was conducted essentially as previously described.[22]

Electrophoretic mobility shift assay

Electrophoretic mobility shift assay (EMSA) was performed using "DIG Gel Shift Kit, 2nd Generation" (Roche Diagnostics GmbH, Mannheim, Germany), according to the manufacturer's protocol. Sequences of oligonucleotides for probes are indicated by the horizontal bar in the relevant figures. Nuclear extracts were prepared, as described previously,[23] from K562 cells or HEK293 human embryonic kidney cells that were transfected with a GATA1-FLAG fusion protein expression vector or its backbone vector.

Promoter/enhancer activity assays

Each target DNA fragment was prepared from genomic DNA from normal volunteers (WT) or patients with CSA (referred to as "GGTA" or "delGATA" in each reporter construct) and was cloned into pGL3basic plasmid (Promega Corporation, Madison, WI, USA). The human ALAS2 proximal promoter region (g.4820_5115, between -267 and +29 from the transcription start site)[16,24] was cloned into the multiple-cloning site of pGL3basic [referred to as pGL3-AEpro(-267)]. A single DNA fragment (5.2 kbp), carrying the ALAS2 proximal promoter, first exon, first intron and the untranslated region of the second exon, was subcloned into the multiple cloning site of pGL3basic [referred to as pGL3-AEpro(-267)+intron1]. A DNA fragment containing the GATA1-binding region in the first intron of the *ALAS2* gene (corresponding to g.7488_7960), which was defined by ChIP-seq analysis,[22] is referred to as the ChIP-peak. The length of the WT ChIP-peak is 473 bp. In addition, a 130-bp fragment containing ALAS2int1GATA, the consensus sequence for the GATA1-binding site in the ChIP-peak, is referred to as ChIPmini. Several deletion mutants of ChIPmini were prepared using pGL3-AEpro(-267)+ChIPmini(WT) as a template. The pGL3-TKpro plasmid was constructed by cloning herpes simplex virus thymidine kinase promoter into the multiple cloning site of pGL3basic plasmid. Each reporter vector and pEF-RL25 were introduced into K562 cells or HEK293 cells. Luciferase activity was determined using a dual-luciferase reporter system (Promega).

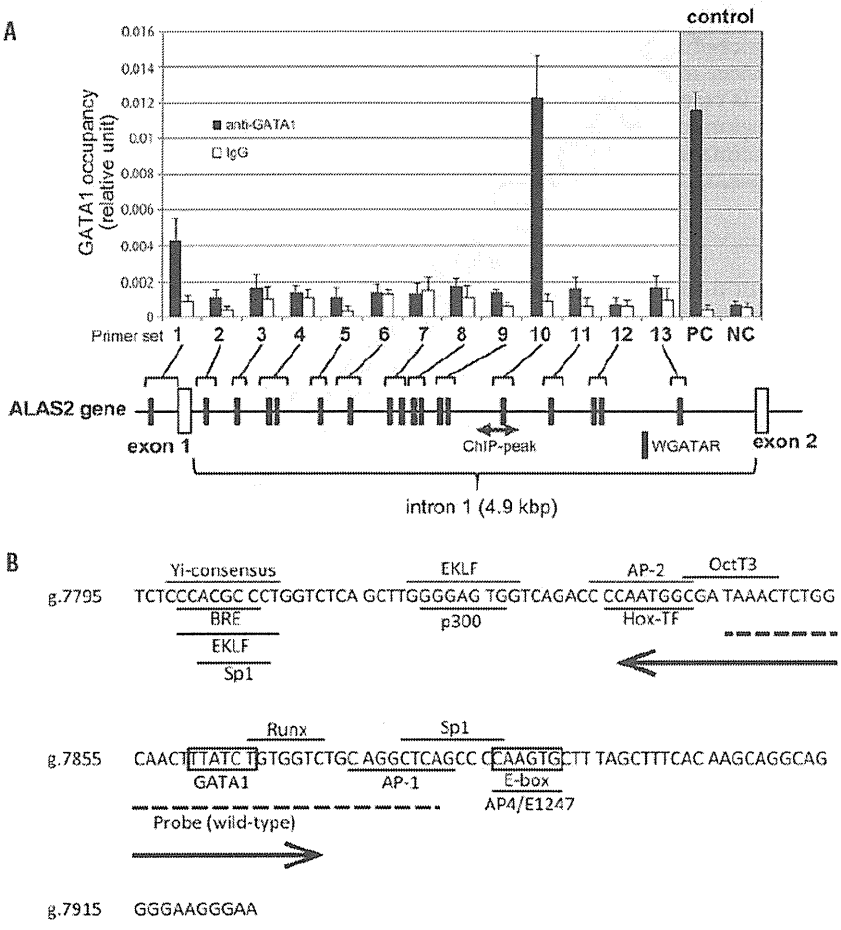


Figure 1. Identification of a functional GATA1 element in the first intron of the *ALAS2* gene. (A) Chromatin immunoprecipitation assay. Fragmented genomic DNA segments were immunoprecipitated with anti-GATA1 antibody or control IgG, and then precipitated fragments were quantified using real-time PCR as described in the *Online Supplementary Methods*. PC or NC indicates positive control or negative control, respectively, for the ChIP assay using anti-GATA1 in K562 cells.[22] One GATA element is present in the proximal promoter region and 17 GATA elements in the first intron (black symbols). The shaded double arrow indicates the region corresponding to ChIP-peak. (B) Nucleotide sequence of ChIPmini. The GATA binding site, ALAS2int1GATA, is located in the center of ChIPmini (boxed). A box also indicates the consensus for E-box that is bound by Scl/TAL1.[22] The sequence of ChIPmini was further analyzed for putative transcription factor binding sites using GeneQuest software (DNASTAR Inc., Madison, WI, USA), and the results are indicated by the horizontal bar. Yi-consensus, Yi transcription factor consensus site;[33] BRE, transcription factor IIB binding site;[34] EKLF, erythroid/Kruppel-like factor consensus site;[35] Sp1, stimulatory protein 1 binding site;[36] P300, P300 transcriptional coactivator consensus site;[37] AP-2, AP-2 beta consensus site;[38] Hox-TF, C1 element binding factor binding site;[39] OctT3, OctT3 binding site;[40] Runx, Runx proteins binding site;[41] AP-1, activator protein 1 binding site;[42] and AP4/E1247, AP4/E1247 binding site.[43] The sequence for the wild-type probe used in the EMSA is indicated by a dashed line. A double arrow indicates the deleted region of the delGATA mutation.

Identification of mutations of the ALAS2 gene

All exons including exon-intron boundaries, the proximal promoter region, and intron 1 and intron 8 of the *ALAS2* gene (GeneBank: NG_8983.1) were directly sequenced according to previously reported methods.[26]

Measurement of ALAS2 mRNA in purified erythroblasts

Total RNA was extracted from glycophorin A-positive bone marrow mononuclear cells, and was used for cDNA synthesis. ALAS2 expression was measured by real-time PCR, and was normalized to that of GAPDH mRNA.

Statistical analysis

Multiple comparisons between groups were made using the Tukey-Kramer test.

Patients

Eleven probands (eight pedigrees) with CSA of unknown cause were selected to determine the nucleotide sequence of the first intron of *ALAS2* gene. In these patients no disease-causative mutation was identified in the coding regions or reported regulatory regions in *ALAS2*, *SLC25A38*, *GLRX5*, *ABCB7*, *PUS1* and *SLC19A2*, which have been reported to be genes causing CSA[11] (see the *Online Supplementary Methods* for full details of the methods).

The genetic analyses performed in this project were approved by the ethical committee of Tohoku University School of Medicine. Blood samples were withdrawn from the probands and the family members after informed consent.

Results

Polymerase chain reaction-based quantitative chromatin immunoprecipitation analysis of the first intron of the ALAS2 gene

To identify the novel regulatory region for ALAS2 tran-

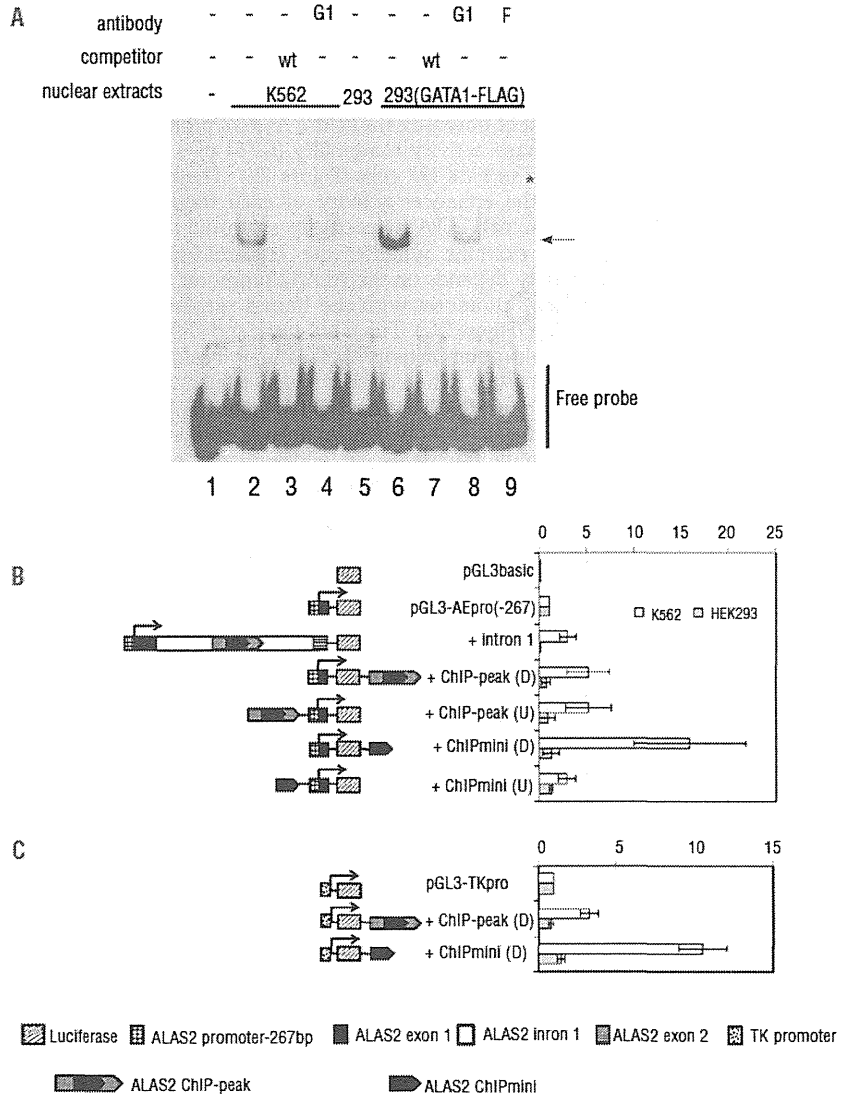


Figure 2. Functional analyses of ChIPmini present in the first intron of the *ALAS2* gene. (A) Electrophoretic mobility shift assay (EMSA). Wild-type (wt) probe was incubated with nuclear extracts prepared from K562 cells (lanes 2-4) or HEK293 cells expressing GATA1-FLAG (lanes 6-9). HEK293 cells were transfected with mock vector (lane 5) or FLAG-fused GATA1 expression vector before preparation of nuclear extracts. The protein-probe complex was detected as a retarded band (arrow). An excess amount of unlabeled probe (lanes 3, 7), anti-GATA1 antibody (G1) (lanes 4, 8) or anti-FLAG antibody (F) (lane 9) was included in the reaction mixture. Lane 1 shows the control without nuclear extracts. The asterisk indicates the super-shifted band (lane 9). (B) Functional analysis of ChIPmini as an enhancer for the *ALAS2* gene. Details of the fragments for each plasmid, such as Intron1, ChIP-peak and ChIPmini, are described in the *Methods* section. Each DNA fragment was inserted upstream of the *ALAS2* proximal promoter or downstream of luciferase cDNA, indicated as (U) or (D), respectively. Results are expressed as a relative activity compared to that of pGL3-AEpro(-267), and are presented as the mean ± standard deviation (SD) of three independent experiments. (C) Functional analysis of ChIPmini as an enhancer for non-erythroid gene promoter. The enhancer activity of the first intron was examined using the herpes simplex virus TK promoter as a non-erythroid promoter. ChIP-peak or ChIPmini was inserted downstream of the luciferase gene of pGL3-TKpro, yielding pGL3-TKpro+ChIP-peak(D) or pGL3-TKpro+ChIPmini(D). Each of these reporter vectors was introduced into K562 cells or HEK293 cells to measure enhancer activity. Results are expressed as a relative activity compared to that of pGL3-TKpro, and are presented as the mean ± SD of three independent experiments.

scription, we first performed ChIP-qPCR analysis in K562 cells to localize the GATA1-binding region of the *ALAS2* gene *in vivo*, which was determined by genome-wide ChIP-seq analysis.[22] In fact, ChIP-qPCR enabled us to examine the GATA1-binding activity of an individual GATA element or two adjacent GATA elements in the first intron of the *ALAS2* gene. Based on a search of NCBI Reference Sequence (NG_8983.1) using SeqBuilder software (DNASTAR Inc., Madison, WI, USA), we identified 17 GATA elements (16 out of 17 GATA elements are present in the antisense orientation) in the first intron of human *ALAS2* (Figure 1A), which is compatible with the previous report.[21] We also included the proximal promoter region that contains a functional GATA-binding site (g.4961_4966).[16] Overall 13 primer sets were designed to amplify the GATA elements located in the proximal promoter region and the first intron of *ALAS2* (Figure 1A and *Online Supplementary Table S1*). Among the 12 primer sets targeting the first intron, using primer set 10, we could amplify genomic DNA that was precipitated with anti-GATA1 antibody at a similar level to that of the positive control, but not with other primer sets. We refer to this region amplified with primer set 10 as ChIPmini (g.7795_7924), the sequence of which is shown in Figure 1B. *In silico* analysis identified only one GATA element (g.7860_7865, boxed in Figure 1B) in ChIPmini, termed ALAS2int1GATA. In addition, primer set 1 which targets the proximal promoter region yielded notable amounts of amplified genome DNA. These results indicate that GATA1 protein bound to the regions amplified with primer sets 1 and 10 in K562 cells; that is, GATA1 protein could bind to the proximal promoter region as well as to ALAS2int1GATA in the first intron of the *ALAS2* gene *in vivo*. Since the GATA element located in the proximal promoter has been well examined *in vitro*,[16] we further determined the functional features of ALAS2int1GATA.

GATA1 protein binds to ALAS2int1GATA located in ChIPmini

We then examined whether GATA1 protein binds to ALAS2int1GATA present in the center of ChIPmini using EMSA (Figure 2A). The WT probe contains ALAS2int1GATA (Figure 1B). The incubation of labeled WT probe with nuclear extracts of K562 cells yielded the retarded band that represents the protein-probe complex (lane 2), whereas this retarded band was undetectable with an excess amount of non-labeled WT probe (lane 3). Moreover, the addition of anti-GATA1 antibody reduced the intensity of the retarded band (lane 4), suggesting that GATA1 protein may bind to the WT probe. In fact, the retarded band was not detected when the labeled probe was incubated with nuclear extracts of mock-transfected HEK293 cells (lane 5). In contrast, the retarded band was observed when the labeled probe was incubated with the nuclear extracts of HEK293 cells expressing FLAG-fused GATA1 (lane 6). Importantly, the retarded band observed in lane 6 was not detectable in the presence of an excess amount of non-labeled probe (lane 7). The formation of the retarded band was partially inhibited by anti-GATA1 antibody (lane 8). Likewise, the inclusion of anti-FLAG antibody (lane 9) resulted in the disappearance of the retarded band and instead generated the super-shifted band (indicated by an asterisk). These results suggest that GATA1 protein binds to the WT probe containing ALAS2int1GATA.

Enhancement of ALAS2 promoter activity by the DNA segment containing ALAS2int1GATA

To examine the functional importance of ALAS2int1GATA in the promoter activity of the *ALAS2* gene (Figure 2B), we constructed the pGL3-AEpro(-267) vector, in which the expression of firefly luciferase gene is controlled under the proximal promoter of the *ALAS2* gene (g.4820_5115). The presence of the first intron of *ALAS2* (pGL3-AEpro(-267)+intron1) increased luciferase activity about 3-fold in K562 cells, whereas luciferase activity was decreased to 10% of pGL3-AEpro(-267) in HEK293 cells. When the ChIP-peak, the region determined by ChIP-seq analysis (g.7488_7960),[22] was present downstream [+ChIP-peak(D)] or upstream [+ChIP-peak(U)] of the ALAS2 proximal promoter, luciferase activity was increased about 5-fold, irrespective of the location, compared to that of pGL3-AEpro(-267) in K562 cells. Moreover, the presence of the ChIPmini fragment downstream of the luciferase gene [+ChIPmini (D)] resulted in a 16-fold increase of luciferase activity. However, when the same fragment was inserted upstream of the ALAS2 promoter [+ChIPmini(U)], luciferase activity increased only 3-fold. Thus, the enhancer activity of the ChIPmini fragment varies, depending on its location. Moreover, among the constructs examined, the ChIPmini fragment showed maximum enhancer activity downstream of the luciferase gene. The ChIP-peak or ChIPmini fragment downstream of the ALAS2 promoter influenced luciferase activity marginally (0.73- or 1.25-fold, respectively) in HEK293 cells (Figure 2B). These results suggest that the enhancer activity of each fragment containing ALAS2int1GATA is specific to erythroid cells.

To examine whether the erythroid-specific enhancer activity depends on the ALAS2 promoter, we replaced the ALAS2 promoter with the herpes simplex virus TK promoter (Figure 2C). The ChIP-peak and ChIPmini enhanced TK promoter activity 3.4- and 9.8-fold in K562 cells, respectively, whereas they did not enhance TK promoter activity in HEK293 cells. These results indicate that the erythroid-specific enhancer is present in the ChIP-peak and ChIPmini fragments. In addition, the erythroid-specific enhancer is functional in the non-erythroid gene promoter.

Identification of mutations in the first intron of the ALAS2 gene in patients with congenital sideroblastic anemia

Considering the newly identified enhancer in the first intron of the *ALAS2* gene, we examined whether some CSA patients carry the mutation in ChIP-peak or ChIPmini of *ALAS2*. We determined the nucleotide sequence of the first intron of *ALAS2* in 11 probands (eight pedigrees), and found two distinct mutations in the newly identified enhancer region in five Japanese patients (three pedigrees). The clinical features and hematologic status of the probands at diagnosis of the disease are summarized in Table 1.

Proband 1 in a pedigree with XLSA

The first male Japanese proband was referred to hospital at the age of 3 months to investigate the cause of his pale face. No problems were reported during the birth. Investigations showed microcytic/hypochromic anemia, an increased concentration of serum iron and raised serum ferritin level. Bone marrow aspiration revealed the presence of ring sideroblasts. Two maternal relatives – male cousins of the proband's mother – have sideroblastic ane-

mia (Figure 3A). The pedigree of this family suggested X chromosome-linked inheritance of the disease. The proband's anemia was not improved by pyridoxine administration (5 mg/kg/day for 3 months), and the boy required once monthly transfusions of one unit of concentrated red blood cells to maintain an adequate hemoglobin level. At the age of 7 months, this proband died of sepsis caused by alpha-streptococcus.

Proband 2 with nfCSA

The second male Japanese proband visited hospital at the age of 4 years because of the paleness of his complexion. Investigations showed microcytic/hypochromic anemia, mild thrombocytosis, and a high serum iron concentration with a normal serum ferritin concentration. Bone marrow aspiration revealed the presence of ring sideroblasts (38% of the erythroblasts). Giant platelets were observed in the bone marrow, although dysplasia of the megakaryocytes was not clear. There was no family history of sideroblastic anemia (Figure 3B).

Proband 3 with nfCSA

The third male Japanese proband was noted to have anemia at the age of 2 years, but details are not available. Without any treatment, serum hemoglobin level was maintained at 70 g/L, and increased to 100 g/L at the age of 10. Accordingly, the proband stopped visiting the hospital. At the age of 19, however, the proband was admitted to hospital because of general fatigue. Investigations revealed microcytic, hypochromic anemia with systemic iron overload. The presence of ring sideroblasts was confirmed in his bone marrow by Prussian blue staining (36% of erythroblasts). Although this proband was treated with pyridoxine (150 mg/day) for 8 months, his anemia did not improve. There was no family history of sideroblastic anemia (Figure 3C).

In proband 1 from the pedigree with XLSA (Figure 3A), we identified a single nucleotide mutation (Figure 4, upper panel, g.7863T>C), which alters the core sequence of ALAS2int1GATA in the antisense strand from GATA to GGTA (referred to as "GGTA mutation"). The same mutation of the *ALAS2* gene was also identified in two cousins of the proband's mother, both of whom were diagnosed as having sideroblastic anemia (Figure 3A). Clinical specimens for genetic analysis were not available from either the parents or the elder brother of proband 1.

The same GGTA mutation was identified at ALAS2int1GATA in proband 2 with CSA (Figure 4, middle panel). There was no known consanguinity between proband 1 and proband 2. Genomic DNA from the parents of proband 2 was not available, because they did not agree to provide their clinical specimens for genetic analysis. Since proband 2 was also noted to have thrombocytosis (Table 1), we searched for a *JAK2* mutation in the genomic DNA extracted from the peripheral blood of this patient. However, no V617F mutation or any missense

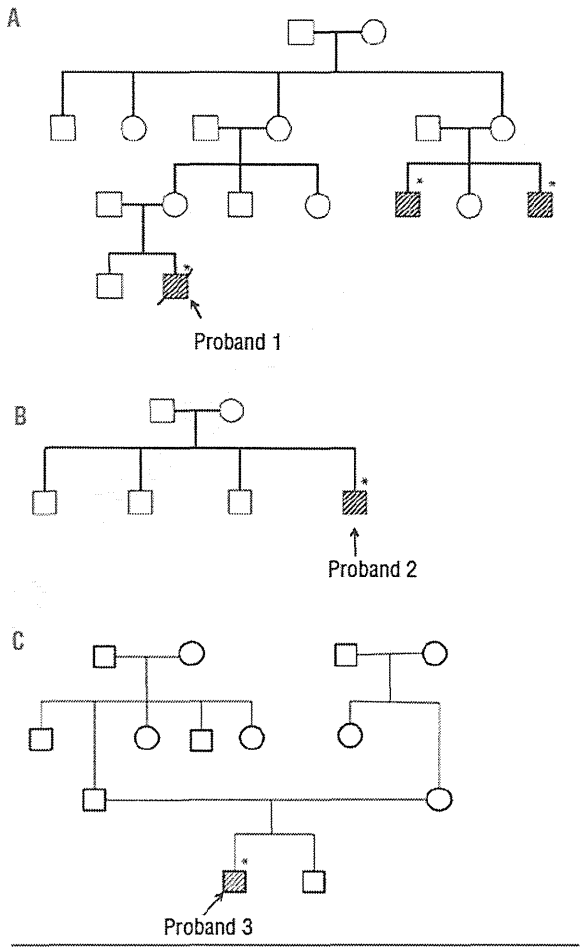


Figure 3. Family trees of three unrelated probands. Family tree of: (A) proband 1 with XLSA, (B) proband 2 with nfCSA, and (C) proband 3 with nfCSA. Shaded boxes indicate affected individuals in each pedigree. Asterisks indicate the individuals in whom a mutation in the first intron of the *ALAS2* gene was detected.

Table 1. **Hematologic status of each proband at diagnosis of the disease.**

	Onset of the anemia	Age at diagnosis of SA	Family history of XLSA	Hb (g/L)	MCV (fL)	MCH (pg)	Platelets (x10⁹/L)	Serum iron (μmol/L)	Ferritin (pmol/L)
Proband 1	4 months	4 months	yes	39 [136-183]	65 [83-101]	18.7 [28-35]	246 [140-379]	63.9 [10.7-37.6]	399.7 [49.4-270]
Proband 2	4 years	4 years	no	84 [126-165]	73.4 [87-104]	22 [29-35]	610 [138-309]	49.1 [12.5-25.0]	670.1 [67.4-725]
Proband 3	2 years	19 years	no	78 [120-165]	73.9 [80-100]	22.2 [28-34]	373 [160-420]	39.6 [14.3-21.5]	2489.7 [40.4-288]

The normal value of each clinical examination is shown in brackets. SA: sideroblastic anemia; XLSA: X-linked sideroblastic anemia; Hb: hemoglobin; MCV: mean corpuscular volume; MCH: mean corpuscular hemoglobin.

mutation in exon 12, each of which is frequently observed in patients with refractory anemia with ring sideroblasts and thrombocytosis (RARS-T),[27] was detected (*data not shown*). Thus, the GGTA mutation at ALAS2int1GATA may be responsible for the sideroblastic anemia in proband 2.

In proband 3 with CSA, a deletion of 35 bp was identified in the first intron of the *ALAS2* gene (Figure 4A, lower panel, g.7836_7870del, referred to as "delGATA mutation"). The delGATA mutation results in the loss of ALAS2int1GATA. However, the delGATA mutation was not identified in the *ALAS2* gene of the parents of proband 3 (*data not shown*). Thus, the delGATA mutation may be a *de novo* mutation or a somatic mutation. Accordingly, we compared the relative ALAS2 mRNA level in the erythroid progenitor cells isolated from proband's bone marrow with those of normal subjects. The ALAS2 mRNA level was more than 7-fold lower in the proband's erythroblasts than in those of three independent, normal subjects (Figure 4B), suggesting that the delGATA mutation may lead to decreased transcription of the *ALAS2* gene.

Lastly, we examined the sequence of the region corresponding to g.7513_8165 of the *ALAS2* gene, which contains ChIPmini, in 103 healthy, Japanese volunteers (44 males and 59 females, total 162 alleles) using PCR followed by direct sequencing. No mutation was found in this region (*data not shown*). In addition, no single nucleotide polymorphism was reported in this GATA element, based on the single nucleotide polymorphism database available at the NCBI home page (*http://www.ncbi.nlm.nih.gov/snp*, current assembly is GRCh37.p5). Thus, the GGTA mutation and delGATA mutation at ALAS2int1GATA may be unique to patients with sideroblastic anemia. Taken together, we suggest that the newly identified mutations at ALAS2int1GATA are responsible for sideroblastic anemia.

The mutation at ALAS2int1GATA impairs GATA1-binding activity and enhancer function

We examined the effect of the GGTA mutation or the delGATA mutation on the binding of GATA1 protein to ALAS2int1GATA using each mutant probe (Figure 5A). The delGATA probe represents the 5'- and 3'-flanking sequences of the deleted 35-bp segment (see Figure 4A). As shown in Figure 5B, the incubation of labeled WT probe with nuclear extracts from HEK293 cells expressing FLAG-fused GATA1

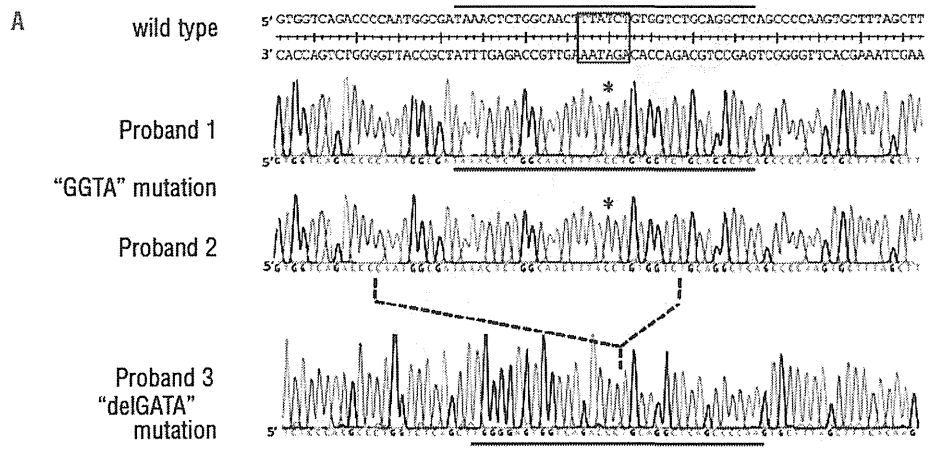


Figure 4. Identification of mutations in the first intron of the *ALAS2* gene in a patient with XLSA and two patients with nfCSA. (A) *ALAS2* mutations in three probands. Upper, middle and lower panels show the sequences of the flanking regions of ALAS2int1GATA (boxed in the wild-type sequence) in the *ALAS2* gene of probands 1, 2 and 3, respectively. Asterisks indicate the T to C transition in the sense strand identified in the *ALAS2* gene of proband 1 and proband 2 with CSA. The broken line between the middle and lower panels indicates the deleted region identified in proband 3 with CSA. The solid horizontal bar in each panel indicates the sequence of the sense strand of each probe used for the EMSA (see Figures 3A and 5B). (B) ALAS2 mRNA expression in erythroblasts of proband 3. ALAS2 mRNA levels were determined in purified erythroblasts isolated from proband 3 and three independent normal individuals using real-time PCR. Results are expressed as the mean ± SD of three independent experiments.

showed a retarded band (lane 3): this band was super-shifted by the addition of anti-FLAG antibody (lane 4), or undetectable with non-labeled WT probe (lane 5), whereas the non-labeled GGTA probe (lane 6) or delGATA probe (lane 7) could not compete for the labeled WT probe. Furthermore, the retarded band was not detectable when labeled GGTA probe (lane 8) or delGATA probe (lane 9) was incubated with the nuclear extracts of HEK293 cells expressing FLAG-fused GATA1. These results suggest that either the GGTA mutation or the delGATA mutation may impair the binding of GATA1 to ALAS2int1GATA.

We then examined the influence of the point mutation or deletion of ALAS2int1GATA on the enhancing activity of the first intron of the *ALAS2* gene (Figure 6A). The GGTA mutation decreased the enhancing activity of the first intron, ChIP-peak or ChIPmini in K562 cells to 17.0%, 18.5% or 12.9%, respectively, of that of the WT construct. The delGATA mutation decreased the enhancing activity of the first intron of *ALAS2*, ChIP-peak or ChIPmini in K562 cells to 10.5%, 15.7% or 12.6%, respectively, of that of the WT construct. In contrast, the relative luciferase activity of the construct carrying each mutation was only marginally different from that of WT intron 1, ChIP-peak or ChIPmini in HEK293 cells (Figure 6A), thereby confirming that ALAS2int1GATA functions as an erythroid-specific enhancer.

There are several potential *cis*-elements at the flanking regions of ALAS2int1GATA, such as EKLF and Sp1, each of which may be involved in the erythroid-specific transcriptional regulation of the *ALAS2* gene.[16,21] We thus analyzed the roles of these *cis*-elements in the enhancer activity of ALAS2int1GATA using deletion mutants at the 5'- or 3'-flanking region of ChIPmini, constructed in pGL3-AEpro(-267)+ChIPmini(D). Deletion of the EKLF1 element at the 5'-flanking region or both E-box and Sp1 elements at the 3'-flanking region did not significantly influence the enhancer activity of ChIPmini (Figure 6B). It should be noted that the Sp1 site overlaps with the 3'-portion of the AP-1 site and the 5'-portion of the E-box (Figure 6C). Moreover, deletion at the 5'-flanking region of ChIPmini ("delEKLF2", "delAP2" and "delOctT3") marginally decreased the enhancer activity (Figure 6B), but the change was not statistically significant. In contrast, deletion of the AP-1 element at the 3'-flanking region ("delAP1" in Figure 6B) significantly decreased the enhancer activity, by about 40% of the activity of ChIPmini(WT). The significant decrease of enhancer activity was observed only in ChIPmini(GGTA), ChIPmini(delGATA) and delAP1, compared to the activity of ChIPmini(WT) ($^{*}P<0.05$ and $^{**}P<0.01$ in Figure 6B). We next constructed another reporter vector that carries an internal deletion of the 5' portion of the AP-1 element with an intact Sp1 site ("lackAP1" in Figure 6B). Internal deletion of the AP-1 element alone in ChIPmini decreased the enhancer activity, although not to a statistically significant degree. Thus, the entire AP-1 element seems to be important for the

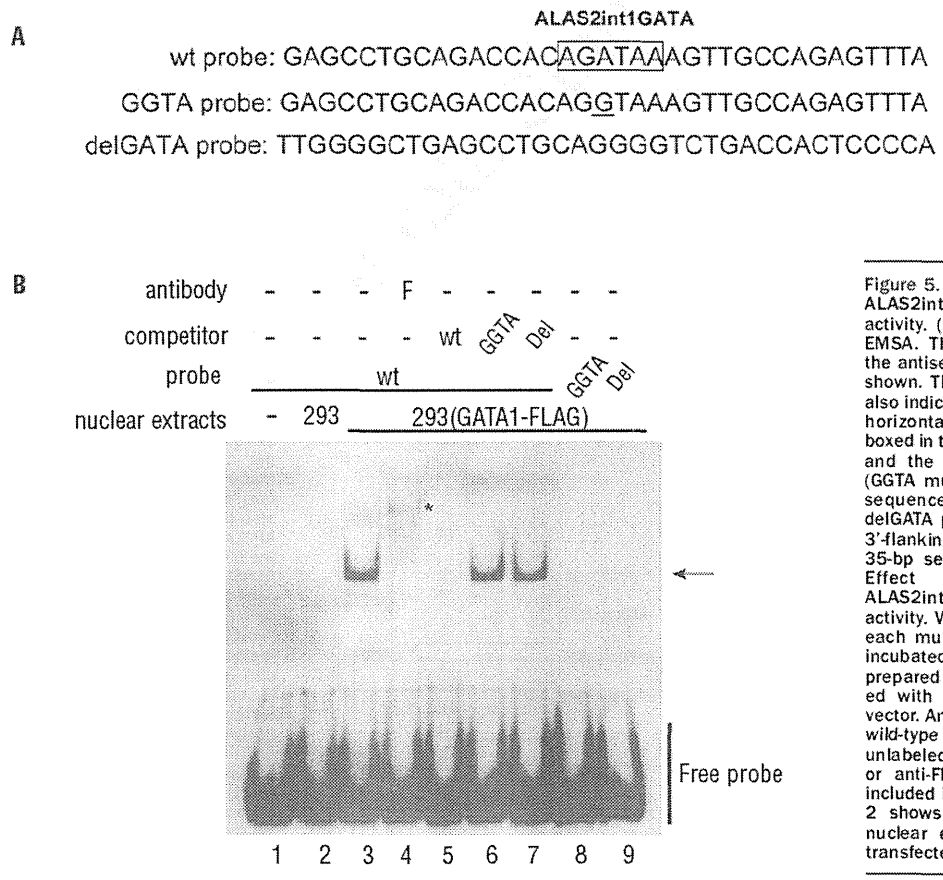
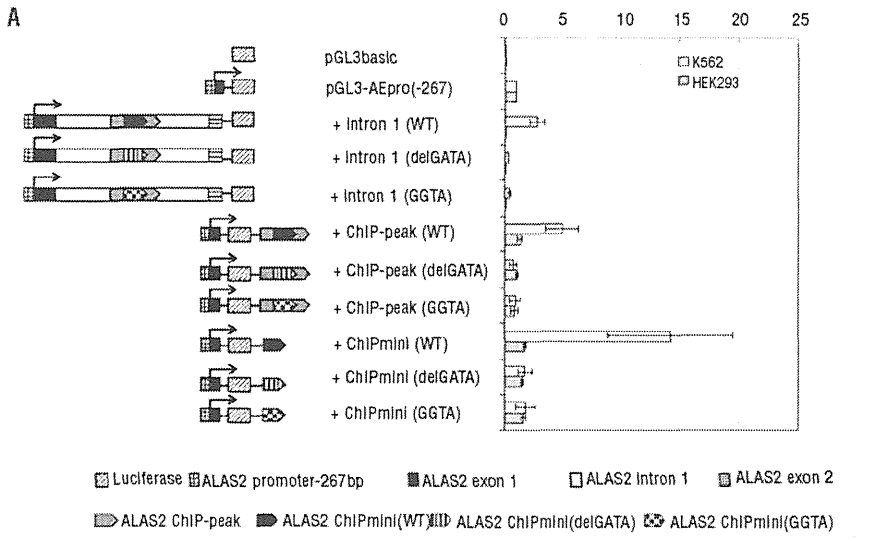
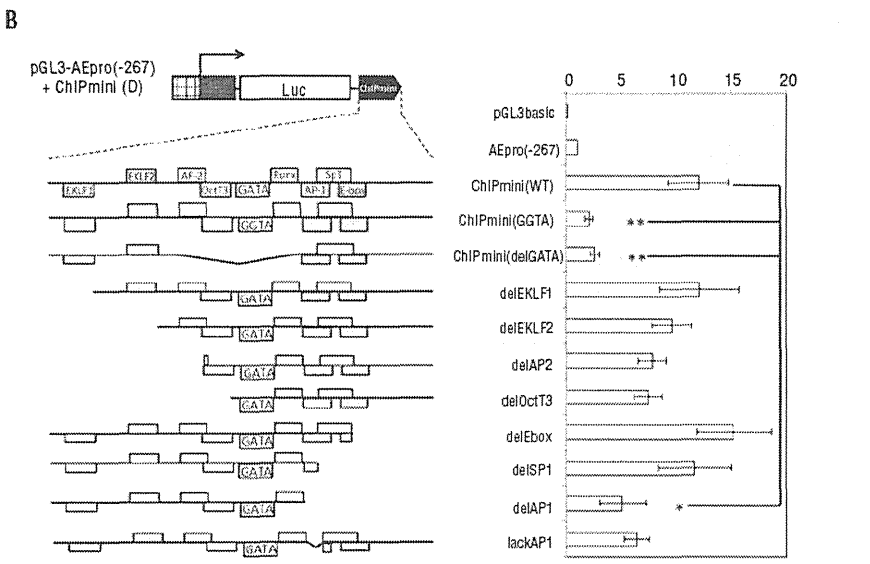
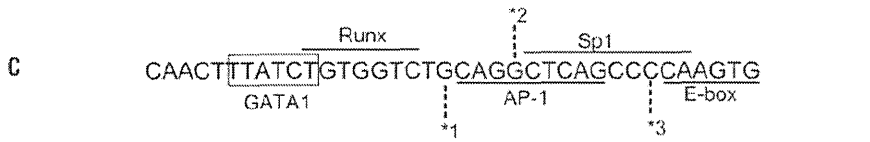


Figure 5. Effects of the mutations of ALAS2int1GATA on GATA1-binding activity. (A) DNA probes used in the EMSA. The nucleotide sequences in the antisense strand of the probes are shown. The position of each probe is also indicated in Figure 1B as the solid horizontal bar. ALAS2int1GATA is boxed in the sequence of the wt probe, and the single nucleotide transition (GGTA mutation) is underlined in the sequence of the GGTA probe. The delGATA probe represents the 5'- and 3'-flanking sequences of the deleted 35-bp segment (see Figure 3B). (B) Effect of each mutation of ALAS2int1GATA on GATA1-binding activity. Wild-type probe (lanes 3-7) or each mutant probe (lanes 8, 9) was incubated with the nuclear extracts prepared from HEK293 cells transfected with the GATA1-FLAG expression vector. An excess amount of unlabeled wild-type probe (lane 5), each of the unlabeled mutant probes (lanes 6, 7), or anti-FLAG antibody (lane 4) was included in the reaction mixture. Lane 2 shows the negative control with nuclear extracts from HEK293 cells transfected with mock vector.







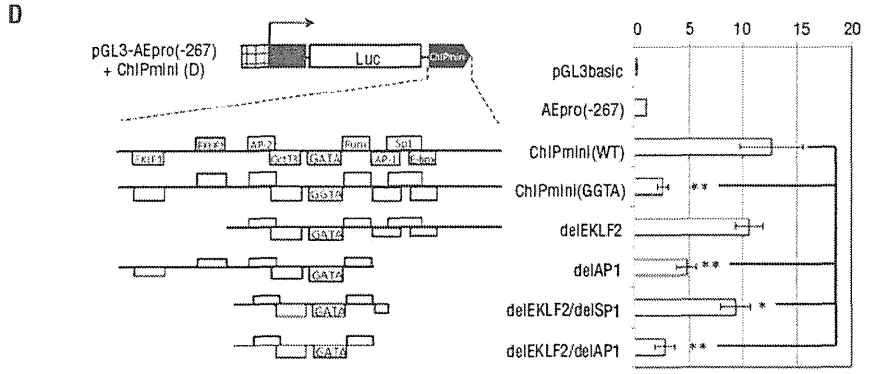


Figure 6. Identification of *cis*-elements essential for the erythroid-specific enhancer activity of ChIPmini. (A) Effect of each mutation of ALAS2int1GATA on the enhancer activity of *ALAS2* ChIPmini. The region corresponding to +intron1, ChIP-peak or ChIPmini, derived from proband 1 or proband 3, was subcloned into pGL3-AEpro(-267) to construct the reporter vector containing the GGTA mutation or the deletion of ALAS2int1GATA, respectively. (B) Effect of the deletion at the 5'- or 3'-flanking region of ALAS2int1GATA on the enhancer activity of ChIPmini. The 5'- and 3'-flanking regions of ALAS2int1GATA contain potential transcription factor-binding sites (*cis*-elements), and a portion of each flanking region was deleted, as schematically shown. The enhancer activity of each deletion mutant was determined in K562 erythroleukemia cells. (C) The nucleotide sequence of the 3'-flanking region of ALAS2int1GATA. Note that the Sp1 site overlaps the AP-1 site and E-box. Each number, *1, *2 or *3, indicates the nucleotide at the 3' end of the deletion mutant, delAP1, delSP1 or delE-box, respectively. Thus, delSP1 also lacks the 3' portion of the AP-1 site. (D) Effect of deletion of the 5'- and 3'-flanking regions of ALAS2int1GATA on the enhancer activity of ChIPmini. The construct, delEKLF2/delSP1, lacks two EKLF sites in the 5'-flanking region and both the Sp1 element and E-box in the 3'-flanking region. The AP-1 element at the 3'-flanking region was deleted from delEKLF2/delSP1, yielding delEKLF2/delAP1. Results are expressed as relative activity compared to that of pGL3-AEpro(-267), and are presented as the mean ± SD of at least three independent experiments.

enhancer activity of ChIPmini (WT) (Figure 6B).

Consequently, we constructed delEKLF2/delSP1 and delELKLF2/delAP1, each of which lacks EKLF elements at the 5'-flanking region and the Sp1 element or the AP-1 element at the 3'-flanking region, respectively (Figure 6D). The deletion mutant, delEKLF2/delSP1, still retained enhancer activity at about 80% of that of ChIPmini(WT), whereas delEKLF2/delAP1 showed decreased enhancer activity similar to the activity of ChIPmini(GGTA). These data indicate that ALAS2int1GATA and its flanking region, especially the AP-1 element, are critically important for the erythroid-specific enhancer activity of ChIPmini.

Taken together, these results suggest that the ChIPmini region acts as an erythroid-specific enhancer for the *ALAS2* promoter, and that both the GGTA mutation and the delGATA mutation represent loss-of-function mutations of ALAS2int1GATA.

Discussion

In the present study, we identified an erythroid-specific enhancer region in the first intron of the human *ALAS2* gene (a 130 bp region referred to as ChIPmini), a region which contains ALAS2int1GATA, a functional GATA1-binding site. We also identified the GGTA mutation and the delGATA mutation at ALAS2int1GATA, each of which is associated with XLSA or CSA. Moreover, we confirmed that each mutation diminished the binding of GATA1 transcription factor to ALAS2int1 (Figure 5B) and decreased enhancer activity of ChIPmini (Figure 6A). Thus, the GGTA mutation and delGATA mutation are loss-of function mutations of the *ALAS2* gene. In fact, the expression of ALAS2 mRNA in bone marrow erythroblasts was lower in proband 3 (Figure 4B) than in normal controls. Thus, each loss-of function mutation may lead to decreased transcription of the *ALAS2* gene, thereby causing sideroblastic anemia in male patients. Such a molecular basis is consistent in part with the lack of pyridoxine responsiveness in these patients (see "Patients" section).

The intronic enhancer, ChIPmini, increased ALAS2 promoter activity most efficiently in erythroid cells when it was present downstream of the promoter (Figure 2B). ChIPmini contains potential *cis*-acting elements, including two EKLF-binding sites, each of which overlaps with the Sp1-binding site or p300-binding site, AP-2 site, OctT3 site Runx site, AP-1 binding site, Sp1 site, and E-box (Figure 1B). Further analysis using deletion mutants of ChIPmini revealed that the potential AP-1 binding site at the 3'-flanking region might be involved in the erythroid-specific enhancer activity of ChIPmini (Figure 6B). These results suggest that ALAS2int1GATA and its 3'-flanking region are essential for the erythroid-specific enhancer activity of ChIPmini. In fact, EKLF[28] and AP-1[29] are involved in erythroid-specific gene expression. It is interesting that the inclusion of the whole first intron of the *ALAS2* gene in a reporter construct resulted in a decrease of ALAS2 promoter activity [11% of pGL3-AEpro(-267)] in non-erythroid HEK293 cells (Figures 2B and 6A). Likewise, the ChIP-peak upstream or downstream of the promoter also reduced the promoter activity in HEK293 cells [73% or 88% of pGL3-AEpro(-267), respectively] (Figure 2B). These results suggest that the first intron of the *ALAS2* gene may contain suppressor element(s) in addition to the erythroid-specific enhancer, although the mechanism of the suppression and the relevant region remain elusive.

We have successfully identified a novel erythroid-specific enhancer for ALAS2 expression, and have identified disease-causative mutations of this enhancer in patients with CSA. Despite the fact that about 50 missense or non-sense mutations of the *ALAS2* gene have been reported as disease-causative mutations in patients with XLSA,[3,30] a mutation in the regulatory region for the transcription of *ALAS2* has rarely been reported to date. Ducamp *et al.* reported a 48-bp deletion of the *ALAS2* gene at the proximal promoter region (c.-91_-44del) in a patient with XLSA, and proposed that the identified deletion would cause XLSA, since the level of ALAS2 mRNA in the proband's bone marrow was lower than that of normal controls.[31] In this context, it has been reported that the deleted region contained a functionally important element for ALAS2 transcription.[16] Bekri *et al.* reported a C-to-G transversion at nucleotide -206 (c.-258C>G) from the transcription start site in the proximal region of the human *ALAS2* gene in patients with XLSA;[24] however, May *et al.* identified this transversion in normal individuals from South Wales at the rate of 0.05, suggesting that this promoter mutation is a polymorphism.[32]

In conclusion, we have identified a novel erythroid-specific enhancer in the first intron of the human *ALAS2* gene, the enhancer function of which may be directed by GATA1 with other transcription factors, such as EKLF and AP-1 binding proteins. Furthermore, we identified the loss-of-function mutation of ALAS2int1GATA, the GATA element within this enhancer, in five of 11 patients with CSA in whom the gene responsible could not be identified. Thus, the intronic region containing ALAS2int1GATA of the *ALAS2* gene should be examined in patients with XLSA or nfCSA in whom the genetic mutation causing the sideroblastic anemia is unknown.

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Information on authorship, contributions, and financial & other disclosures was provided by the authors and is available with the online version of this article at www.haematologica.org.

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Review Article

Pathophysiology and genetic mutations in congenital sideroblastic anemia

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Abstract Sideroblastic anemias are heterogeneous congenital and acquired disorders characterized by anemia and the presence of ringed sideroblasts in the bone marrow. Congenital sideroblastic anemia (CSA) is a rare disease caused by mutations of genes involved in heme biosynthesis, iron–sulfur [Fe-S] cluster biosynthesis, and mitochondrial protein synthesis. The most common form is X-linked sideroblastic anemia, due to mutations in the erythroid-specific δ-aminolevulinate synthase (*ALAS2*), which is the first enzyme of the heme biosynthesis pathway in erythroid cells. Other known etiologies include mutations in the erythroid specific mitochondrial transporter (*SLC25A38*), adenosine triphosphate (ATP) binding cassette B7 (*ABCB7*), glutaredoxin 5 (*GLRX5*), thiamine transporter *SLC19A2*, the RNA-modifying enzyme pseudouridine synthase (*PUS1*), and mitochondrial tyrosyl-tRNA synthase (*YARS2*), as well as mitochondrial DNA deletions. Due to its rarity, however, there have been few systematic pathophysiological and genetic investigations focusing on sideroblastic anemia. Therefore, a nationwide survey of sideroblastic anemia was conducted in Japan to investigate the epidemiology and pathogenesis of this disease. This review will cover the findings of this recent survey and summarize the current understanding of the pathophysiology and genetic mutations involved in CSA.

Key words ALAS2, congenital sideroblastic anemia, heme, iron, mitochondria.

Sideroblastic anemias are a group of disorders that have common features of mitochondrial iron accumulation in bone marrow erythroid precursors (ringed sideroblasts), ineffective erythropoiesis, increased levels of tissue iron, and varying proportions of hypochromic erythrocytes in the peripheral blood.[1,2] In adults, these syndromes are commonly found in association with myelodysplastic syndrome, in which their pathogenesis is obscure. Sideroblastic anemia also occurs after exposure to certain drugs or alcohol and in association with copper deficiency.[1,3] In contrast, congenital forms of sideroblastic anemia have been reported, which involve mutations in genes associated with mitochondrial iron-heme metabolism.[1,2,4,5]

Given that congenital sideroblastic anemias (CSA) are very uncommon genetic disorders, and their genetic and pathological features have not yet been fully elucidated, we recently conducted a nationwide survey of congenital and acquired sideroblastic anemia in Japan.[6] Here, we present a review of the findings of that recent survey and also the current understanding of the pathophysiology and genetic mutations involved in CSA.

Genetic features and pathophysiology of CSA

The pathogenesis of most sideroblastic anemias is not well understood.[1,5,7] Because abnormal accumulation of intra-mitochondrial iron is one of the peculiar characteristics of this disease, a great deal of attention has been focused on alterations in genes related to intra-mitochondrial heme-iron metabolism, including (i) heme biosynthesis; (ii) iron–sulfur [Fe-S] cluster biosynthesis; and (iii) mitochondrial protein synthesis (Fig. 1). Several genes responsible for CSA have been identified (Table 1).[1,2,4,5,8] Here, we describe the suggested roles of these genes in the pathophysiology of sideroblastic anemia.

Defects of heme biosynthesis

In the most frequent form of X-linked sideroblastic anemia (XLSA), the defect involves the δ-aminolevulinate synthase (5-aminolevulinate synthase 2; ALAS2), which is located at Xp11.21 and encodes the first enzyme of the heme biosynthetic pathway in erythroid cells.[9,10] The reaction involves condensation of glycine with succinyl-coenzyme A to yield 5-aminolevulic acid (ALA), which requires pyridoxal 5'-phosphate (PLP; vitamin B6) as a cofactor to stimulate the enzymatic activity of ALAS2.[11] It was reported that ALAS2 activity is decreased in the bone marrow of CSA patients, suggesting that impaired heme biosynthesis may induce the onset of sideroblastic anemia. With the exception of several nonsense mutations in clinically affected female carriers, the patients are male and present at a wide variety of ages,[12] but typically before the age of 40.[5] Mutations of ALAS2 in XLSA are heterogeneous, and are usually missense mutations of conserved amino acids that lead to loss of function.[2,4,5,7,8] To date, more than 60 different mutations in ALAS2 have been reported in patients with XLSA.[2,5,13] Missense

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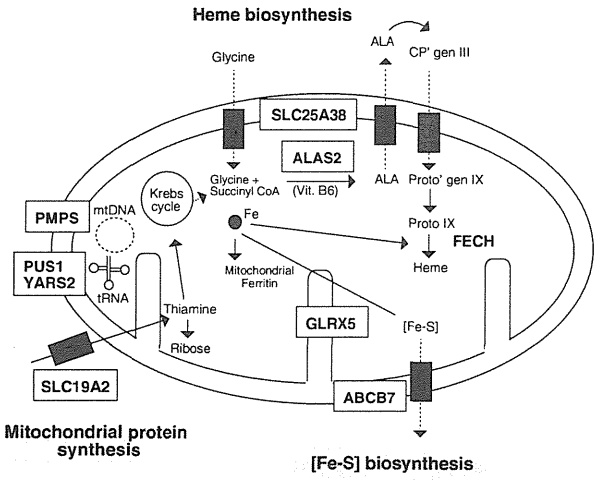

Fig. 1 Genes mutated in various types of congenital sideroblastic anemia. The pathogenic genes can be classified as those involved in (i) heme biosynthesis; (ii) iron–sulfur [Fe-S] cluster biosynthesis; and (iii) mitochondrial protein synthesis. ABCB7, adenosine triphosphate (ATP) binding cassette B7; ALA, 5-aminolevulinic acid; ALAS2, erythroid-specific δ-aminolevulinate synthase (5-aminolevulinate synthase 2); FECH, ferrochelatase; GLRX5, glutaredoxin 5; mtDNA, mitochondrial DNA; PLP, pyridoxal 5′-phosphate (vitamin B6); PMPS, Pearson marrow–pancreas syndrome; PUS1, pseudouridine synthase 1; SLC19A2, high-affinity thiamine transporter SLC19A2; SLC25A38, mitochondrial carrier protein SLC25A38; YARS2, tyrosyl-tRNA synthase, mitochondrial 2.

mutations of ALAS2 are commonly observed from exon 5 through 11, encompassing exon 9, which contains the lysine responsible for PLP binding.[14] In contrast, mutations of ALAS2 regulatory region, such as the promoter[15] and intron 1,[16,17] have also been reported, which lead to decreased ALAS2 expression.[15–17] Taken together, the decrease in ALAS2 expression level, as well as the defects in catalysis, substrate or cofactor affinity, and protein processing of ALAS2 have been implicated in the pathogenesis of XLSA, and supplementation with PLP may contribute to mitigation of these impairments.

Recently, it has become evident that there is a subset of patients with severe hypochromic anemia resembling XLSA but lacking *ALAS2* mutations and who are unresponsive to PLP. Guernsey *et al.* carried out genome-wide scans in subjects with familial or sporadic CSA, and identified several mutations of the *SLC25A38* gene,[18] which was confirmed in a subsequent study.[19] The patterns of mutation vary, including nonsense, frameshift, splice acceptor site, and missense mutations, and the mode of inheritance is autosomal recessive. *SLC25A38* encodes an erythroid-specific protein of the inner mitochondrial membrane, and has been predicted to be involved in mitochondrial import of glycine, which is essential for ALA synthesis,[18] suggesting that dietary supplementation with glycine may ameliorate SLC25A38 anemia. More importantly, because ALAS2 catalyzes glycine and succinyl-CoA to ALA, supplementation with ALA may lead to improvement of CSA involving *ALAS2* and *SLC25A38* mutations.

Defects of [Fe-S] *cluster biosynthesis*

Identification of the genetic basis of XLSA pointed to impaired heme synthesis as the key pathogenetic mechanism of sideroblastic anemia. In contrast, two subsequently recognized forms, X-linked sideroblastic anemia with ataxia (XLSA/A) and glutaredoxin 5 (GLRX5) deficiencies, are due to mutations of proteins involved in the [Fe-S] pathway, an important pathway of mitochondrial iron utilization (Fig. 1).[20]

X-linked sideroblastic anemia with ataxia is a rare type of sideroblastic anemia inherited in an X-linked manner similar to XLSA. XLSA/A patients present with mild anemia and elevated red blood cell protoporphyrin IX (PPIX), with motor delay and evidence of spinocerebellar dysfunction, including early onset ataxia associated with severe cerebellar hypoplasia.[21,22] Systemic iron overload has not been reported in this disease. From the results of molecular analysis, XLSA/A is due to mutations in the adenosine triphosphate (ATP) binding cassette B7 (*ABCB7*) gene, which is located at Xp13.3 and encodes an essential component of the [Fe-S] cluster machinery. Mutations are missense and loss of function,[21] whereas nonsense mutations have not been reported, perhaps because a complete loss of *ABCB7* would be incompatible with life, as occurs in *Abcb7*-deficient mice.[23] The ABCB7 protein is localized to the inner mitochondrial membrane, and it has been suggested to be involved in transport of the [Fe-S] cluster to the cytosol based on analysis of the yeast ortholog *ATM1*.[24] When ABCB7 activity is impaired, iron

Table 1 Genetic features of congenital sideroblastic anemia

	Inheritance	Chromosome	Gene	Mutation type	Treatment
XLSA	X-linked	Xp11.21	ALAS2	M, N	Pyridoxine
XLSA/A	X-linked	Xp13.3	ABCB7	M	–
SA/GLRX5	AR	14q32.13	GLRX5	M, S	?
SA/SLC25A38	AR	3p22.1	SLC25A38	M, N, S	?
PMPS	Maternal†	Mitochondria	Mitochondrial	D	–
TRMA	AR	1q24.2	SLC19A2	M, N	Thiamine
MLASA/PUS1	AR	12q24.33	PUS1	M, N	–
MLASA/YARS2	AR	12p11.21	YARS2	M	–

†Sporadic cases are also reported. D, deletion; M, missense; MLASA, mitochondrial myopathy and sideroblastic anemia; N, nonsense; PMPS, Pearson marrow–pancreas syndrome; S, splicing; TRMA, thiamine-responsive megaloblastic anemia; XLSA, X-linked sideroblastic anemia; XLSA/A, X-linked sideroblastic anemia with ataxia.

remains trapped in mitochondria and the level of [Fe-S] cluster-dependent enzyme activities is decreased in the affected cells. Furthermore, it was shown that the interaction of ABCB7 with ferrochelatase (FECH) increases the activity of FECH, which is the final enzyme in the heme biosynthetic pathway. Therefore, similar to *ALAS2*- and *SLC25A38*-associated sideroblastic anemia, a decreased level of heme likely contributes to the pathogenesis of ring sideroblast formation.

Another type of sideroblastic anemia is due to mutations of *GLRX5*, a gene that encodes a mitochondrial protein involved in [Fe-S] cluster biogenesis.[25] The single patient described had a homozygous GLRX5 splicing mutation that strongly reduced mRNA and protein levels,[4,25] and presented mild anemia until the fifth decade, when anemia worsened, with a relatively low number of ring sideroblasts in the bone marrow, and other iron-related complications were diagnosed. Wingert *et al.* previously showed GLRX5 to be mutated in the zebrafish hypochromic microcytic anemia mutant *shiraz*.[26] The study demonstrated that loss of the Fe-S cluster in the iron-regulatory protein 1 (IRP1) blocked ALAS2 translation by binding to the iron-responsive element (IRE) located in the 5′-untranslated region of ALAS2 mRNA. These findings were substantially confirmed in fibroblasts derived from patients or in erythroblasts in which GLRX5 expression had been reduced by siRNAs.[27]

Therefore, the pathological link between [Fe-S] biosynthesis defect, caused by ABCB7 and GLRX5 mutations, and sideroblastic anemia may be attributable to a secondary defect of heme biosynthesis in erythroblasts.

Abnormal mitochondrial protein synthesis

Pearson marrow–pancreas syndrome (PMPS) is a rare syndromic disorder, presenting with sideroblastic anemia, accompanied by metabolic acidosis, ataxia, and endocrine pancreas dysfunction.[28] The disease is usually fatal, and patients die during infancy. Erythropoiesis in PMPS is macrocytic and non-megaloblastic. In addition to ringed sideroblasts, bone marrow aspiration specimens characteristically show vacuolization of early erythroid and myeloid progenitors. PMPS is usually sporadic, but individuals with PMPS may be born to mothers with milder mitochondrial phenotypes. The mechanism of ring sideroblast formation in PMPS remains unclear. Nearly half of all patients with PMPS can be shown to have heteroplasmy for a 4977 bp deletion in the mitochondrial genome,[29] and the canonical deletion may result in deficiency of mitochondrially encoded subunits of respiratory complex I (NADH dehydrogenase), complex IV (cytochrome *c* oxidase), and complex V (ATP synthase), as well as mt-tRNA genes. Iron should be in the reduced state (Fe^{2+}) when incorporated into PPIX by FECH in the final step of heme synthesis.[9] It is speculated that cytochrome *c* oxidase functions to keep iron in the reduced state, and therefore the defect of cytochrome *c* enzymatic activity may result in defects of heme biosynthesis, leading to the appearance of ringed sideroblasts.[1] As indicated in toxic sideroblastic anemia associated with the antibiotic chloramphenicol, an inhibitor of bacterial ribosomal translation, which is closely structurally related to mammalian mitochondrial ribosomes, mutations of mt-tRNAs may also lead to global mitochondrial impairment by suppressing translation of multiple mitochondrial DNA-encoded proteins.[1,5,30]

The association of CSA with defective mitochondrial protein expression can be seen most directly in the mitochondrial myopathy with lactic acidosis and ringed sideroblasts (MLASA) phenotype, which results from mutations in genes encoding either of two proteins, pseudouridine synthase (*PUS1*) or mitochondrial tyrosyl-tRNA synthase (*YARS2*). Patients with MLASA due to *PUS1* mutation typically present with lactic acidosis and mitochondrial myopathy associated with decreases in respiratory complexes I and IV.[31,32] PUS1 functions in pseudouridine modification of tRNAs.[31] Pseudouridine is known to affect the structure of tRNA and to strengthen base pairing. Thus, failure of pseudouridine modification may lead to aberrant translation. In contrast, MLASA due to *YARS2* has been identified in patients of Lebanese origin.[33,34] Although the mechanisms by which ringed sideroblasts occur in these cases have not been elucidated mainly due to the limited number of patients, it is speculated that the reduced aminoacylation activity of mutant YARS2 enzyme may lead to decreased mitochondrial protein synthesis, resulting in mitochondrial respiratory chain dysfunction.[33] In both PUS1 and YARS2 mutations, there may be defects in the mitochondrial respiratory chain, likely generating an environment that retards iron access to FECH in the reduced form, similar to PMPS.

Thiamine-responsive megaloblastic anemia (TRMA) represents sideroblastic anemia with systemic symptoms, including diabetes and deafness. Mutations in the high-affinity thiamine transporter SLC19A2 are the basis of the disorder, which is responsive to thiamine sup plementation.[35,36] Although it is not clear how mutations of *SLC19A2* are involved in sideroblast formation, it is speculated that the impairment of thiamine-dependent generation of succinyl-CoA, which is required for heme synthesis, is the cause of the ringed sideroblast abnormality, but thiamine is also an essential cofactor in the de novo synthesis of ribose, which is essential for protein synthesis. The link between TRMA and mitochondrial protein synthesis must be experimentally validated in future studies.

Epidemiological characteristics of causative genes for CSA

We recently conducted a nationwide survey of sideroblastic anemia in Japan to investigate the epidemiology and pathogenesis of the disease.[6] As of 31 January 2012, detailed data were available for 148 sideroblastic anemia patients, including 18 cases of CSA, as well as secondary cases such as myelodysplastic syndrome.[6] With the extension of the study, we further identified one additional case of CSA. Among 19 cases of CSA, 11 cases were diagnosed as XLSA due to *ALAS2* missense mutation (n = 10) and presumably due to a partial duplication of the coding region in the *ALAS2* exon 11 (n = 1). Whereas the causative gene mutations were not identified for the remaining eight cases based on the standard detection strategies, novel mutations in the intronic enhancer region of the *ALAS2* gene have recently been identified in four of the eight cases.[17] Thus, in total, 15 of 19 cases (78.9%) were considered to be attributable to mutations

involving the *ALAS2* gene in the Japanese population. In contrast, Bergmann *et al.* recently reported genetic analysis of a relatively large cohort (n = 83) of CSA cases in the USA.[8] In that study, mutations of *ALAS2*, *SLC25A38*, mitochondria DNA, and *PUS1* were identified in 37%, 15%, 2.5%, and 2.5%, respectively,[8] and the remaining cases (43%) remained genetically undefined.[8] The observation that XLSA due to *ALAS2* mutation represents the most common form of CSA is consistent with our observations. The most significant difference, however, was that whereas mutations of the *SLC25A38* gene were frequently found in their study,[8] it was not detectable in our study.[6] To our knowledge, mutations of the *SLC25A38* gene have not been reported in Asia, although they have been widely reported in the USA, Canada, and Europe.[8,18,19] Taken together, it is suggested that the causative genes for CSA differ among races and regions.

Conclusion

Several recent advances have been made with regard to applying molecular genetics to the study of CSA, represented by next-generation sequencing techniques. Although CSA is a rare hematological disorder, it is important to identify the gene mutations that are responsible for genetically unclassified CSA cases. Analyzing their function, based on model organisms ranging from yeast to zebrafish to mice, will improve our knowledge of mitochondrial iron metabolism.

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Brief Report

RED CELLS, IRON, AND ERYTHROPOIESIS

Variant ALDH2 is associated with accelerated progression of bone marrow failure in Japanese Fanconi anemia patients

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Key Points

- We found the defective ALDH2 variant is associated with accelerated progression of BMF in Japanese FA patients.
- The data support the view that aldehydes are an important source of genotoxicity in the human hematopoietic system.

Fanconi anemia (FA) is a severe hereditary disorder with defective DNA damage response and repair. It is characterized by phenotypes including progressive bone marrow failure (BMF), developmental abnormalities, and increased occurrence of leukemia and cancer. Recent studies in mice have suggested that the FA proteins might counteract aldehyde-induced genotoxicity in hematopoietic stem cells. Nearly half of the Japanese population carries a dominant-negative allele (rs671) of the aldehyde-catalyzing enzyme *ALDH2* (acetaldehyde dehydrogenase 2), providing an opportunity to test this hypothesis in humans. We examined 64 Japanese FA patients, and found that the *ALDH2* variant is associated with accelerated progression of BMF, while birth weight or the number of physical abnormalities was not affected. Moreover, malformations at some specific anatomic locations were observed more frequently in *ALDH2*-deficient patients. Our current data indicate that the level of ALDH2 activity impacts pathogenesis in FA, suggesting the possibility of a novel therapeutic approach. (*Blood.* 2013;122(18):3206-3209)

Introduction

Fanconi anemia (FA) is a genomic instability disorder with phenotypes including progressive bone marrow failure (BMF), developmental abnormalities, and increased occurrence of leukemia and cancer.[1] To date, 16 genes have been implicated in FA, and their products form a common DNA repair network ("FA pathway").[2,3] Because FA cells are hypersensitive to DNA interstrand crosslinks (ICLs), the FA pathway has been considered to be involved in the repair of ICLs.[2,3] However, it remains unclear what type of endogenous DNA damage is repaired through the FA pathway. Recent studies have suggested that FA cells are also sensitive to aldehydes,[4] which may create DNA adducts including ICLs or DNA-protein crosslinks. Furthermore, double knockout mice deficient in *Fancd2* and *Aldh2*, but neither of the single mutant mice, display an accelerated development of leukemia and BMF.[5,6] On the other hand, Fanc-deficient mice in general do not fully recapitulate the human FA phenotype, including overt BMF.[7] Thus, the role of aldehydes in the pathogenesis of human FA is still uncertain.

ALDH2 deficiency resulting from a Glu504Lys substitution (rs671, hereinafter referred to as the A allele) is highly prevalent in East Asian populations. The A allele (Lys504) acts as a dominant negative, since the variant form can suppress the activity of the Glu504 form (G allele) in GA heterozygotes by the formation of heterotetramers.[8] Individuals with the A variant experience flushing when drinking alcohol, and have an elevated risk of esophageal cancer with habitual drinking.[9] Because the frequency of the A allele is close to 50% in the Japanese population at large, some Japanese FA patients are expected to be deficient in *ALDH2*. We thus set out to determine the *ALDH2* status in a collection of Japanese FA patients.

Study design

The onset of BMF was defined according to the criteria used in the International Fanconi Anemia Registry (IFAR) study.[10] Criteria for diagnosis of aplastic anemia and other conditions are described in supplemental Methods (available on the *Blood* Web site). We observed physical abnormalities characteristic of FA, including skin abnormalities (hyperpigmentation and café au lait spots), low birth weight, growth defects, and malformations affecting

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Table 1. Summary of genotypes and clinical characteristics of the patients studied

		ALDH2 genotype		
	Total	GG	GA	AA
No. of cases	64	36	25	3
Mutated FA gene*				
FANCA	39	26†	11	2
FANCG	15	7	8	—
FANCI	2	—	2	—
FANCM	1	—	1	—
FANCP	2	—	1	1
Unknown	5	3	2	—
Disease				
Aplastic anemia	2	2	—	—
Severe aplastic anemia	40	21	19	—
MDS/AML	22	13	6	3‡
Tongue cancer	2	1	1	—
Median months of onset (range)				
BMF	52 (0-297)	72 (27-297)	28 (7-87)	0 (0-7)
MDS/AML	118 (4-384)	156 (61-384)	85 (41-192)	4 (4-12)
No. of cases with SCT (%)	58 (91)	33 (92)	23 (92)	2 (67)
Median months at SCT (range)	118 (12-448)	130 (52-448)	86 (28-248)	25 (13-36)

–, no case was found.

*Mutations found in the patients were listed in supplemental Table 1. Some of them were presumptive because their functional significance has not been determined.

†Somatic mosaicism due to reversion was confirmed in 2 cases and suspected in 1 case.

‡In these cases, onset of severe aplastic anemia and MDS was essentially simultaneous.

skeletal systems and deep organs. Extensive malformation was defined as the involvement of at least 3 sites including at least 1 deep organ.[11] Mutation analysis of *FANCA/FANCC/FANCG* genes,[12] *ALDH2* genotyping,[13] multiplex ligation-mediated probe amplification (MLPA) test for *FANCA* (Falco), and whole-exome sequencing (WES)[14] were done as previously described. Details are provided as supplemental Methods. Development of BMF or acute myeloid leukemia (AML)/myelodysplasia (MDS) was analyzed by the Kaplan-Meier method or the cumulative incidence method,[15,16] respectively, since competing events (eg, death and stem cell transplantation [SCT]) existed in AML/MDS but not in BMF. This study was approved by the Research Ethics Committee of the Tokai University Hospital and Kyoto University. We obtained family informed consent from all subjects involved in this work in accordance with the Declaration of Helsinki.

Results and discussion

All of the patients in this study (n = 64; supplemental Table 1) were referred to the Tokai University Hospital because of pancytopenia, in some cases with MDS or leukemia. The clinical diagnosis of FA was made based on clinical presentation and diepoxybutane (DEB)-induced chromosome fragility tests in peripheral blood lymphocytes,[17] except for 3 cases in which the DEB test was negative due to *FANCA* reversion mosaicism (supplemental Tables 1-2). Most of the patients underwent allogeneic SCT, indicating that our patients probably represent an FA population with relatively severe hematologic symptoms.

To determine which FA gene was mutated in each of these patients, we applied combinations of polymerase chain reaction–based methods (n = 26), the MLPA test for *FANCA* mutations (n = 44), and WES (n = 29). In our WES analysis, >90% of the 50-Mb target sequences were analyzed by >10 independent reads (data not shown). Fifty-nine patients were found to have a mutation in FA genes in at least 1 allele, but 5 of them were mutation-free in the known 16 FA genes, even after WES (Table 1; supplemental Table 1). These unclassified cases might be caused by large deletions or intronic mutations that are difficult to detect with these methods,[18] or possibly mutations in a novel FA gene.

We determined the *ALDH2* genotype in our series of 64 patients (Table 1; supplemental Table 1). The distribution of the *ALDH2* variant alleles appeared not significantly different from the reported allele frequencies in the healthy Japanese population.[13] The occurrence of leukemia and/or MDS was also not significantly different between patients with GA and GG genotypes. Strikingly, however, we found that progression of BMF was accelerated in heterozygous carriers of the variant A allele compared with homozygous GG patients (Figure 1A-B). Moreover, the 3 individuals carrying AA alleles developed MDS with BMF at a very young age (Figure 1A-B). None of these 3 patients belonged to FA-D1 or FA-N, the FA subgroups with severe symptoms.[19,20] Patient number 3 had biallelic frameshift mutations (S115AfsX11) in *FANCP/SLX4*. By contrast, of the FA-P patients that have previously been reported, none have displayed particularly severe symptoms.[21-23]

FA is a heterogeneous disorder, and our cohort of patients is quite heterogeneous in terms of complementation groups and types of mutations (Table 1). To reduce some of the variability, we selected only the *FANCA* patients having nonsense, frameshift, or large deletion mutations identified at both alleles, (n = 12; supplemental Table 1), and repeated the analysis. A patient with probable *FANCA* reversion (patient number 55) was excluded. In this subset of patients, a highly significant statistical difference was reproduced in BMF progression (Figure 1C) but not in AML/MDS development (data not shown).

We could not detect any significant difference in terms of the percentage of birth weight (Figure 1D) or number of physical abnormalities (Figure 1E) that correlated with the *ALDH2* genotypes. However, a significant difference was observed in the incidence of each class of malformations in the case of radial, cardiovascular, skeletal, or kidney anomalies, and in the incidence of extensive malformation (Figure 1F).

In conclusion, our current data indicate that endogenous aldehydes are an important source of genotoxicity in the human hematopoietic system, and the FA pathway counteracts them. If the FA pathway is compromised, hematopoietic stem cells (HSCs) likely accumulate aldehyde-induced DNA damage, resulting in BMF due to p53/p21-mediated cell death or senescence.[6,24] Consistent with this model, a recent study showed that the HSCs in *aldh2/fancd2* double knockout mice accumulate more DNA damage than HSCs in either of the single knockout mice.[6] Because some *ALDH2*-proficient FA patients developed BMF early, other modifier genes or environmental factors might affect levels of aldehydes or other genotoxic substances. Interestingly, our data predict that Japanese FA patients in general develop BMF at an earlier age compared with patients of other ethnic origins. We need to establish a Japanese FA registry similar to IFAR to test whether this is true or not. Finally, it seems worth considering *ALDH2* agonists such as Alda-1 as protective drugs against BMF in FA patients. Alda-1 can stimulate the enzymatic activity of both the normal and variant ALDH2,[25] suggesting that Alda-1 or a similar drug could be beneficial even for *ALDH2*-proficient FA cases.