complex (lane 2), whereas this retarded band was undetectable with an excess amount of non-labeled wild-type 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 wild-type 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 with 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 with asterisk). These results suggest the binding of GATA1 protein to the wild-type 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 ALAS2 gene (Fig. 2B), we constructed pGL3-AEpro(-267) vector, in which the expression of firefly luciferase gene is controlled under the proximal promoter of ALAS2 gene (g.4820_5115). The presence of the first intron of ALAS2 gene (pGL3-AEpro(-267)+intron1) increased the luciferase activity about three-fold in K562 cells, whereas the luciferase activity was rather 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] is present at the downstream (+ChIP-peak(D)) or the upstream (+ChIP-peak(U)) of ALAS2 proximal promoter, the luciferase activity was increased about five-fold, irrespective of the location, compared to that of pGL3-AEpro(-267) in K562 cells. Moreover, the presence of ChIPmini fragment at the downstream of luciferase gene (+ChIPmini (D)) resulted in the 16-fold increase of the luciferase activity. However, when it was inserted at the upstream of the ALAS2 promoter (+ChIPmini(U)), the luciferase activity increased only three-fold. Thus, the enhancer activity of the ChIPmini fragment may vary, depending on its location. Moreover, the ChIPmini fragment showed maximum enhancer activity at the downstream of the luciferase gene

among the constructs examined. On the other hand, the ChIP-peak or ChIPmini fragment at the downstream of ALAS2 promoter marginally influenced the luciferase activity (0.73- or 1.25-fold, respectively), in HEK293 cells (Fig. 2B). These results suggest that the enhancer activity of each fragment containing ALAS2int1GATA is specific to erythroid cells.

Then, to examine whether the erythroid-specific enhancer activity depends on ALAS2 promoter, we replaced ALAS2 promoter with the herpes simplex virus TK promoter (Fig. 2C). The ChIP-peak and ChIPmini enhanced TK promoter activity 3.4- and 9.8-fold in K562 cells, respectively, whereas they could 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 ALAS2 gene in patients with CSA**

Considering the newly identified enhancer in the first intron of ALAS2 gene, we examined whether some of CSA patients carry the mutation in ChIP-peak or ChIPmini of

the ALAS2 gene. We determined the nucleotide sequence of the first intron of ALAS2 gene in eleven probands (eight pedigrees), and found two distinct mutations at newly identified enhancer region in five Japanese probands (three pedigrees). Clinical features of the representative proband are following, and hematologic status of each proband on diagnosis of the disease is summarized in Table 1.

**Proband 1 in a pedigree with XLSA**: First male Japanese proband visited the hospital at 3 months after his birth for checking the cause of his pale face. No trouble was reported during the birth. Clinical examinations revealed the presence of microcytic/hypochromic anemia, increased serum iron concentration and serum ferritin level. Bone marrow aspiration revealed the presence of ring sideroblasts. There are two sideroblastic anemia patients in the maternal relatives, male cousins of the proband's mother (Figure 3A). The pedigree tree of this family suggested the X chromosome linked inheritance of the disease. The anemia of this proband was not improved by pyridoxine administration (5 mg/kg/day for three months), and the transfusion of one unit of concentrated red blood cells was required once a month to maintain the hemoglobin level. At age seven months, this proband died of sepsis caused by alpha-streptococcus.

**Proband 2 with nfCSA:** Second male Japanese proband visited the hospital at age of 4 years, because of the paleness of his complexion. Clinical examinations revealed microcytic/hypochromic anemia with mild thrombocytosis and high serum iron concentration with the normal serum ferritin concentration. Bone marrow aspiration revealed the presence of ring sideroblasts (38% of the erythroblasts). Giant platelets were observed in bone marrow, although dysplasia of the megakaryocytes was not clear. There was no family history of sideroblastic anemia for this proband (Figure 3B).

**Proband 3 with nfCSA:** Third male Japanese proband was pointed out his anemia at age of 2 years, but details were not available. Without any treatment, serum hemoglobin level was maintained at 70 g/L, and it was increased to 100 g/L at age 10. Accordingly, the proband stopped to visit the hospital. At age 19, however, the proband admitted to the hospital because of general fatigue. Clinical examination 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 eight months, his anemia was not improved. There was no family history of sideroblastic anemia for this proband

(Figure 3C).

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

Moreover, the same GGTA mutation was identified at ALAS2int1GATA in proband 2 with CSA (Fig. 4, middle panel), while the consanguinity between proband 1 and proband 2 was not identified. The genomic DNA from parents of proband 2 was not available, because they did not agree to provide their clinical specimens for genetic analysis. Since the thrombocytosis was pointed out for this proband (Table 1), we also examined the presence of JAK2 mutation in the genomic DNA extracted from the peripheral blood of this proband. However, V617F mutation or any missense mutation in

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

In proband 3 with CSA, a deletion of 35 bps was identified in the first intron of ALAS2 gene (Fig. 4A, lower panel, g.7836_7870del; referred 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 lower by more than seven folds in proband's erythroblasts than those of three independent normal subjects (Fig. 4B), suggesting that the delGATA mutation may lead to the decreased transcription of ALAS2 gene.

Lastly, we examined the sequence of the region corresponding to g.7513_8165 of ALAS2 gene, which contains ChIPmini, in 103 Japanese healthy volunteers (44 males

and 59 females, total 162 alleles) using PCR followed by direct sequencing. As a result, 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 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 the GATA1-binding activity and the 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 (Fig. 5A). The delGATA probe represents the 5'- and 3'-flanking sequences of the deleted 35-bp segment (see Fig. 4A). As shown in Figure 5B, the incubation of labeled wild-type probe with nuclear extracts from HEK293 cells expressing FLAG-fused GATA1 showed

retarded band (lane 3), and such retarded band was super-shifted by the addition of anti-FLAG antibody (lane 4), or was undetectable with non-labeled wild-type probe (lane 5), whereas non-labeled GGTA probe (lane 6) or delGATA probe (lane 7) could not compete for the labeled wild-type probe. Furthermore, 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.

Then, we examined the influence of the point mutation at or the deletion of ALAS2int1GATA on the enhancing activity of the first intron of ALAS2 gene (Fig. 6A). The GGTA mutation also decreased the enhancing activity of the first intron, ChIP-peak or ChIPmini in K562 cells to 17.0%, 18.5% or 12.9% of that of wild-type construct, respectively. 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% of that of wild-type construct, respectively. In contrast, the relative luciferase activity of the construct carrying each mutation was only marginally different from that of wild-type

intron 1, ChIP-peak or ChIPmini in HEK293 cells (Fig. 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 ALAS2 gene.[16,21] We thus analyzed the roles of these cis-elements for 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 EKLF1 element at 5'-flanking region or both E-box and Sp1 element at the 3'-flanking region did not significantly influence the enhancer activity of ChIPmini (Fig. 6B). It should be noted that the Sp1 site overlaps with the 3'-portion of the AP-1 site and the 5'-portion of E-box (Fig. 6C). Moreover, deletion at the 5'-flanking region of ChIPmini ("delEKLF2", "delAP2" and "delOctT3") marginally decreased the enhancer activity (Fig. 6B), but the change was not statistically significant. In contrast, the deletion of the AP-1 element at the 3'-flanking region ("delAP1" in Fig. 6B) significantly decreased the enhancer activity, about 40% activity of ChIPmini(WT). Thus, 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 Fig.6B). We next constructed another reporter vector that carries an internal deletion of the 5' portion of the AP-1 element with the intact Sp1 site ("lackAP1" in Fig. 6B). Internal deletion of the AP-1 element alone in ChIPmini decreased the enhancer activity, but the degree of the decrease was not statistically significant. Thus, the entire AP-1 element seemed to be important for the enhancer activity of ChIPmini (WT) (Fig 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 (Fig. 6D). The deletion mutant, delEKLF2/delSP1, still retained enhancer activity at about 80% of that of ChIPmini(WT), whereas delEKLF2/delAP1 showed the decreased enhancer activity that was 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 erythroid-specific enhancer activity of ChIPmini.

Taken together, these results suggest that ChIPmini region acts as an

erythroid-specific enhancer for ALAS2 promoter, and both GGTA mutation and delGATA mutation represent a loss-of-function mutation of ALAS2int1GATA.

**Discussion**

In the present study, we identified erythroid-specific enhancer region in the first intron of human ALAS2 gene (referred as ChIPmini of 130 bp), the region of 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 (Fig. 5B) and decreased enhancer activity of ChIPmini (Fig. 6A). Thus, the GGTA mutation and delGATA mutation are loss-of function mutations of ALAS2 gene. In fact, the lower expression level of ALAS2 mRNA was observed in bone marrow erythroblasts prepared from proband 3 (Fig. 4B), compared with normal controls. Thus, each loss-of function mutation may lead to the decreased transcription of 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 at downstream of the promoter (Fig. 3B).

ChIPmini contains potential cis-acting elements, including two EKLF-binding sites, each of which is overlapping 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 (Fig. 2B). 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 (Fig. 6B). These results suggest that ALAS2int1GATA and its 3'-flanking region are essential for erythroid-specific enhancer activity of ChIPmini. In fact, EKLF[28] and AP-1[29] are involved in the erythroid-specific gene expression. It is of interest that the inclusion of the whole first intron of ALAS2 gene in a reporter construct resulted in the decrease of ALAS2 promoter activity (11% of pGL3-AEpro(-267)) in non-erythroid HEK293 cells (Figs. 3B and 6A). Likewise, the ChIP-peak at the upstream or downstream of the promoter also reduced the promoter activity in HEK293 cells (73% or 88% of pGL3-AEpro(-267), respectively) (Fig. 3B). These results suggest that the first intron of ALAS2 gene may contain suppressor element(s) in addition to the erythroid-specific enhancer, while the mechanism of the suppression and the relevant region remain to be elusive.

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

In conclusion, we have identified the novel erythroid-specific enhancer in the

first intron of 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 within eleven patients with congenital sideroblastic anemia, in whom responsible gene 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 responsible gene for sideroblastic anemia is unknown.

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**Authorship and Disclosures**

K.F. and H.H. designed the study; R.K. and H.I. recruited the patients; K.K., T.F. and K.F. performed experiments; K.K., K.F., T.F., R.K. and H.I. prepared figures and tables; K.F., K.K., T.F., R.K., H.I. H.H. and S.S. wrote the paper.

There are no relevant conflicts of interest to disclose.

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