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The carboxyl-terminal region of erythroid-specific 5-aminolevulinate synthase acts as an intrinsic modifier for its catalytic activity and protein stability

Senkottuvelan Kadirvel[a], Kazumichi Furuyama[a], Hideo Harigae[b], Kiriko Kaneko[c], Yoshiko Tamai[d], Yoji Ishida[e], and Shigeki Shibahara[a]

[a]Department of Molecular Biology and Applied Physiology; [b]Department of Hematology and Rheumatology; [c]Endocrinology and Applied Medical Science, Tohoku University School of Medicine, Sendai, Japan; [d]Division of Transfusion Medicine, Hirosaki University Hospital, Hirosaki, Japan; [e]Hematology and Oncology, Internal Medicine, Iwate Medical University School of Medicine, Morioka, Japan

Erythroid-specific 5-aminolevulinate synthase (ALAS2) is essential for hemoglobin production, and a loss-of-function mutation of ALAS2 gene causes X-linked sideroblastic anemia. Human ALAS2 protein consists of 587 amino acids and its carboxyl(C)-terminal region of 33 amino acids is conserved in higher eukaryotes, but is not present in prokaryotic ALAS. We explored the role of this C-terminal region in the pathogenesis of X-linked sideroblastic anemia. In vitro enzymatic activity was measured using bacterially expressed recombinant proteins. In vivo catalytic activity was evaluated by comparing the accumulation of porphyrins in eukaryotic cells stably expressing each mutant ALAS2 tagged with FLAG, and the half-life of each FLAG-tagged ALAS2 protein was determined by Western blot analysis. Two novel mutations (Val562Ala and Met567Ile) were identified in patients with X-linked sideroblastic anemia. Val562Ala showed the higher catalytic activity in vitro, but a shorter half-life in vivo compared to those of wild-type ALAS2 (WT). In contrast, the in vitro activity of Met567Ile mutant was about 25% of WT, while its half-life was longer than that of WT. However, in vivo catalytic activity of each mutant was lower than that of WT. In addition, the deletion of 33 amino acids at C-terminal end resulted in higher catalytic activity both in vitro and in vivo with the longer half-life compared to WT. In conclusion, the C-terminal region of ALAS2 protein may function as an intrinsic modifier that suppresses catalytic activity and increases the degradation of its protein, each function of which is enhanced by the Met567Ile mutation and the Val562Ala mutation, respectively. © 2012 ISEH - Society for Hematology and Stem Cells. Published by Elsevier Inc.

5-Aminolevulinate synthase (ALAS) is the first and rate-limiting enzyme in the heme biosynthetic pathway [1]. There are two isozymes of ALAS in higher eukaryotes, ALAS1 and ALAS2. ALAS1 (alternatively, ALAS-N) is expressed ubiquitously in all types of nucleated cells, and expression of ALAS2 (or ALAS-E) is restricted in erythroid cells and essential for hemoglobin production during erythroid differentiation [1]. Both ALAS1 and ALAS2, which are encoded by the distinct nuclear genes, function in mitochondria [2,3], and the amino-terminal

region of each isozyme acts as a targeting signal for mitochondrial translocation [4–6]. The remaining regions of ALAS protein consist of a core catalytic region and a carboxyl terminal (C-terminal) region, and the catalytic region is conserved among several species [7]. In addition, the C-terminal region of 33 amino acids (positions 555–587), which is encoded by the 11th exon of the human ALAS2 gene, is well conserved in higher eukaryotes, but the equivalent region is not present in bacterial ALAS [7]. It is conceivable that the C-terminal region of mammalian ALAS2 protein might have an important regulatory role in heme biosynthesis.

The human ALAS2 gene that is mapped on X chromosome consists of 11 exons [8], and a genetic mutation of the ALAS2 gene causes X-linked sideroblastic anemia (XLSA) [9,10] or X-linked dominant protoporphyria [11]. To the best of our knowledge, >50 different mutations of

the ALAS2 gene have been identified in about 100 pedigrees with XLSA [12–14]. Reported mutations in patients with XLSA are distributed from the 5th exon to the 11th exon of the human ALAS2 gene, but only four mutations were detected in 11th exon [14–17]. In the case of X-linked dominant protoporphyria, two different frame-shift mutations have been identified in the 11th exon of the ALAS2 gene in two independent probands [11]. These frame-shift mutations cause deletions of 19 and 21 amino acids at the C-terminal end of ALAS2, both of which are accompanied by replacement of the C-terminal end with one unrelated amino acid and an unrelated peptide of 23 amino acids, respectively. Using recombinant proteins expressed in *Escherichia coli*, those authors provided evidence that deletion of 19 or 21 amino acids at C-terminal end increased the catalytic activity of ALAS2, suggesting that the C-terminal region can inhibit the enzymatic activity of ALAS2 [11]. Recently, it was also reported that the substitution (Tyr586Phe) at the penultimate amino acid of the C-terminal of ALAS2 increased its catalytic activity in vitro, which might be related to the severe phenotype of congenital erythropoietic porphyria [18]. Interestingly, such gain-of-function mutations of the ALAS2 gene were solely identified within the C-terminal region of ALAS2 protein. However, it is still unclear how the C-terminal region of ALAS2 is involved in the regulation of ALAS2 function in vivo.

Here, we report novel missense mutations in the 11th exon of the ALAS2 gene in independent probands with XLSA. Based on in vitro and in vivo functional studies of these mutants, as well as a C-terminal deletion mutant, we provide evidence that the C-terminal region of human ALAS2 protein reduces its catalytic activity and protein stability in mitochondria.

Case reports

Case 1

Japanese male proband presented with microcytic hypochromic anemia (hemoglobin: 8.1 g/dL; mean corpuscular volume: 57.7 fL) at age 14 years. Serum ferritin, serum iron, and total iron binding capacity were 222.7 ng/mL, 242 μg/dL, and 279 μg/dL, respectively. Proband's mother and maternal uncles had mild anemia, but they did not receive any medication for anemia.

Bone marrow examination of the patient showed erythroid hyperplasia (myeroid to erythroid ratio [M:E] = 0.45), with ringed sideroblasts comprising >10% of nucleated cells. Pyridoxine treatment (80 mg/d) was started, and the hemoglobin concentration gradually increased from 7.3 g/dL to 12.0 g/dL after 14 months.

Case 2

Japanese male proband was admitted to the hospital at age 36 years because of microcytic hypochromic anemia

(hemoglobin: 6.5 g/dL; mean corpuscular volume: 64.4 fL) with systemic iron overload (ferritin: 2581.4 ng/mL). Anemia was pointed out before he was school age, but he did not receive any medication for anemia. Prussian blue staining of bone marrow cells revealed the presence of ring sideroblasts in the proband, and the diagnosis of sideroblastic anemia was established. Pyridoxine treatment (60 mg/d) was started when hemoglobin was 5.4 g/dL, then anemia was improved after 1 month to 9.9 g/dL hemoglobin. Although pyridoxine treatment was continued for an additional 4 months, the hemoglobin level did not exceed 10 g/dL.

Materials and methods

Reagents

Chemical reagents were purchased from Sigma-Aldrich (St Louis, MO, USA), Nacalai Tesque (Kyoto, Japan), or Wako Pure Chemicals (Osaka, Japan). Restriction enzymes and modifying enzymes used for construction of each plasmid were purchased from New England Biolabs (Ipswich, MA, USA), unless otherwise noted. ExTaq DNA polymerase and PrimeStar Max DNA polymerase were purchased from Takara Bio Inc. (Shiga, Japan) and were used for polymerase chain reaction (PCR) and site-directed mutagenesis, respectively. Protein concentration was measured with Bio-Rad Protein assay reagent (Bio-Rad Laboratories Inc., Hercules, CA, USA) or Pierce 660 nm Protein Assay Reagent (Thermo Scientific, Rockford, IL, USA) using bovine serum albumin as a standard. Sodium dodecyl sulfate polyacrylamide electrophoresis (SDS-PAGE) and Western blot analysis were performed as described previously [19]. Prestained XL-ladder broad range (APRO Science, Tokushima, Japan) was loaded as a size marker for SDS-PAGE and Western blot analysis.

Identification of ALAS2 mutations

Genetic analyses performed in this project had been approved by the ethical committee of Tohoku University School of Medicine. Blood samples were drawn from the probands and the family members after informed consent. Genomic DNA was then extracted from them using QIAamp DNA Blood Midi Kit (Qiagen GmbH, Hilden, Germany). All exons including exon-intron boundaries, the proximal promoter region, and the erythroid enhancer in intron 8 of ALAS2 gene were amplified using ExTaq DNA polymerase. Sequences of primers and the condition for PCR were reported previously [20], except for an antisense primer for exon 5 and a primer pair for the erythroid-specific enhancer region in intron 8. The sequence of antisense primer for exon 5 used is (5′-TCATCTCCTCTGGCCACTGC-3′). For the amplification of the erythroid-specific enhancer in intron 8, the following primers were used: sense, 5′-GGTACCACTCGCATCCCACTGCA GAG-3′and antisense, 5′-GGTACCACACAGCCAAAGGCCTT GCC-3′. Each amplified DNA fragment was electrophoresed on 1% agarose gel in TAE buffer and stained with ethidium bromide. DNA fragment was excised from the gel for purification using QIAquick Gel Extraction Kit (Qiagen GmbH). Purified DNA fragment was directly sequenced using BigDye terminator v1.1 cycle sequencing kit and ABI 3100 Genetic Analyzer (Applied Biosystems, Foster City, CA, USA). The same primers were used for PCR and direct sequencing analysis. Sequencing results were

analyzed using Lasergene software (DNASTAR Inc., Madison, WI, USA), and the mutation of ALAS2 gene was confirmed by repeated amplification and direct sequencing.

Expression and purification of recombinant ALAS2 proteins
Complementary DNA for human mature ALAS2 that lacks the amino-terminal region was amplified with PrimeStar Max DNA polymerase (Takara Bio Inc.) using the following primers (sense, 5′-GGTGGTCATATGATCCACCTTAAGGCAACAAAGG-3′; antisense, 5′-GGCATAGGTGGTGACATACTG-3′), each of which was phosphorylated at its 5′ end beforehand. Amplified complementary DNA (cDNA) was digested with NdeI restriction enzyme, and was cloned between NdeI site and blunt ended SapI site of pTXB1 expression vector (New England Biolabs). Resulting plasmid, named as pTXB1-AEm, expresses human mature ALAS2 in *E. coli* as a fusion protein with Intein tag and Chitin binding domain at its C-terminal end. Using pTXB1-AEm as a template, each mutation or deletion was introduced using PrimeStar Max site-directed mutagenesis kit (Takara Inc.). The sequences of primers used for mutagenesis are available upon request. After the amplification of cDNA or mutagenesis, the sequence of mature ALAS2 cDNA and the junction sequence for fusion protein was confirmed by DNA sequencing before use. These expression vectors were used for transformation of the *E. coli* strain, BL21(DE3). Expression and purification of recombinant proteins were performed according to manufacturer's instruction for Impact System (New England Biolabs), with minor modifications. Briefly, expression of recombinant proteins was induced in *E. coli* with 0.1 mM isopropyl β-D-1-thiogalactopyranoside at 25°C for overnight. The isopropyl β-D-1-thiogalactopyranoside–treated cells were collected by centrifugation and resuspended with lysis buffer (20 mM Tris-HCl [pH 8.5], 300 mM NaCl, 1 mM EDTA, 0.1% Triton X-100, 1 mM phenylmethanesulfonyl fluoride, 1 μg/mL antipain, 1 μg/mL pepstatin, and 1 μg/mL leupeptin). After sonication and centrifugation, cleared cell lysates were incubated with Chitin beads for 1 hour at 4°C, and then washed with wash buffer (20 mM Tris-HCl [pH 8.5], 500 mM NaCl, 1 mM EDTA, and 0.1% Triton X-100). To obtain a tag-free recombinant mature ALAS2 protein, on-column cleavage was induced with 50 mM dithiothreitol in wash buffer at room temperature for 16 hours. After the elution from the column, each recombinant protein was dialyzed against wash buffer before use. Purity of each recombinant protein was examined using SDS-PAGE, followed by staining with Quick-CBB PLUS (Wako Pure Chemical). Enzymatic activity of each recombinant protein was measured according to the protocol described previously [21]. Student's *t* test was performed for statistical analysis.

Expression of wild-type or mutant ALAS2 protein
in eukaryotic cells
The plasmid "pGEM-AET," which carries cDNA for full-length ALAS2 tagged with FLAG at its C-terminal, was described previously [22]. Site-directed mutagenesis was performed by PrimeStar Max mutagenesis kit (Takara Inc.) using pGEM-AET as a template to obtain cDNA encoding each FLAG-tagged mutant. In addition, cDNA encoding FLAG-tagged luciferase protein was constructed by replacing ALAS2 cDNA in pGEM-AET with amplified luciferase cDNA derived from pGL3 basic (Promega Corporation, Madison, WI, USA).

For establishing the stable transformants in which expression of FLAG-tagged ALAS2 protein or FLAG-tagged luciferase protein is inducible with tetracycline, cDNA for each protein was cloned into pcDNA5/FRT/TO vector (Invitrogen Corporation, Carlsbad, CA, USA). The resulting cDNA construct was then co-introduced with pOG44 vector into Flp-In T-REx 293 cells (Invitrogen), derived from human embryonic kidney cells (HEK293). After transfection, cells were incubated with 100 μg/mL Hygromycin B (Wako Pure Chemicals) and 15 μg/mL Blasticidin (Invitrogen). At least three independent clones, which were resistant to Hygromycin B and sensitive to Zeocin (Invitrogen), were selected and expanded for subsequent experiments. This phenotype of a given clone confirmed the integration of each cDNA expression cassette into the expected site in the genome of Flp-In T-REx 293 cell line.

For the determination of protein stability, expression of wild-type ALAS2 or mutant ALAS2 was induced by the addition of tetracycline into the culture medium (final concentration of 1 μg/mL) for 48 or 72 hours, and then the culture medium was replaced with fresh complete medium containing tetracycline with or without 10 μM cycloheximide. At 0, 3, 6, 9, and 12 hours after incubation, cells were harvested and lysed in RIPA buffer (10 mM Tris-HCl [pH 7.2], 150 mM NaCl, 1% TritonX-100, 1 mM sodium fluoride, 0.4 mM Na_3VO_4, 10 mM N-ethylmaleimide, 1 mM phenylmethanesulfonyl fluoride, 2 μg/mL leupeptin, and 2 μg/mL aprotinin). Cell lysates were centrifuged at 13,200g for 10 minutes at 4°C, and the supernatants were used for SDS-PAGE. Expression of FLAG-tagged ALAS2 protein was detected by Western blot analysis with anti-FLAG M2 monoclonal antibody (Sigma-Aldrich) as a first antibody. For normalization of loaded samples, glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was detected with anti-GAPDH monoclonal antibody (MAB374; Millipore Corporation, Billerica, MA, USA) as a first antibody. For a second antibody, horseradish peroxidase–conjugated anti-mouse IgG (NA931Vl GE Healthcare, UK Limited, Buckinghamshire, UK) was used. Intensity of each band was measured using ImageJ software (available at http://rsb.info.nih.gov/ij/). The intensity of each band for FLAG-tagged ALAS2 was normalized with that of GAPDH, and the normalized intensity of FLAG-tagged ALAS2 at each time point was compared with that of the sample harvested at 0 hour. We repeated this series of experiments three times for each clone, and an average of these results was used for determination of the half-life of each protein.

The catalytic activity of each mutant protein was also evaluated by comparing the accumulation of porphyrins in Flp-In T-Rex 293 cells that expressed wild-type or mutant ALAS2 cDNA in an inducible manner. For this assay, cells of low-passage numbers (between passage 5 and passage 15) were used for obtaining reproducible results. To induce expression of wild-type ALAS2 or mutant ALAS2 protein in isolated cell lines, cells were treated for 60 hours with tetracycline at a suitable concentration (12.5–50 ng/mL), depending on cell lines. Then, cells were washed with phosphate-buffered saline twice and collected in the sample tube. Flp-In T-REx 293 cells, which express FLAG-tagged luciferase protein in an inducible manner, were also treated with tetracycline as a negative control. Cells were separately collected for Western blot analysis and RNA preparation. Real-time PCR analysis was performed as described previously [23]. Remaining cells were collected by centrifugation and then packed cells were exposed to ultraviolet light for detection of porphyrins.

Table 1. Summarized features of recombinant ALAS2 proteins

| Recombinant protein | In vitro enzymatic activity (nmol ALA/mg protein/h) | | | Half-life in HEK293 cells (h) | Porphyrin accumulation in HEK293 cells |
	Without PLP (% of wild-type)	With 200 µM PLP (% of wild-type)	Ratio with/without PLP		
Wild-type	14,824 ± 754 (100%)	27,627 ± 713 (100%)	1.86	7.8	+++
Val562Ala	22,324 ± 1,555 (150.6%)	32,300 ± 709 (116.9%)	1.44	5.3	++
Met567Ile	5,653 ± 897 (38.1%)	6,975 ± 299 (25.2%)	1.23	>12	±
Ser568Gly*	(19.5%)*	(31.6%)*	2.51*	>12	±
delC33	15,769 ± 382 (106.4%)	53,066 ± 1,843 (192.1%)	3.37	>12	++++

*Data with GST-fused Ser568Gly protein taken from reference [15].

Results

Identification of novel mutations of ALAS2 gene

Analyzing the genomic DNA extracted from the proband of case 1, we identified the c.T1685C mutation in the 11th exon of ALAS2 gene (Supplementary Figure E1A, upper panel; online only, available at www.exphem.org). This transition results in an amino acid substitution at the 562nd residue of ALAS2 protein from valine to alanine (Val562Ala). The same mutation was identified in one allele of the proband's mother (Supplementary Figure E1A, middle panel; online only, available at www.exphem.org), and the proband's father does not carry this mutation (Supplementary Figure E1A, lower panel; online only, available at www.exphem.org), indicating the X-linked inheritance of this mutation. For the proband of case 2, the c.G1701C transversion was identified in exon 11 of ALAS2 gene (Supplementary Figure E1B; online only, available at www.exphem.org), the mutation of which results in an amino acid substitution at the 567th residue from methionine to isoleucine (Met567Ile).

To exclude the possibility that these mutations represent single nucleotide polymorphisms, we examined the 11th exon of ALAS2 gene in 96 Japanese healthy volunteers (57 male and 39 female subjects, with the total allele number of 135) using PCR followed by direct sequencing. As a result, no base change was found in the 11th exon of ALAS2 gene in these subjects, suggesting that the mutation found in each proband might not represent single nucleotide polymorphism. It is therefore conceivable that either the c.T1685C or c.G1701C mutation might be responsible for XLSA.

Enzymatic activities of mutant ALAS2 proteins in vitro

Wild-type ALAS2 or each mutant ALAS2 protein was expressed in *E. coli* and purified as a tag-free protein. The combination of pTXB1 expression vector and IMPACT system allowed us to obtain a tag-free/C-terminal intact recombinant protein. Indeed, modified Coomassie Brilliant Blue staining of the gel after SDS-PAGE revealed that the purity of each prepared protein was >95% (data not shown). These recombinant proteins were suitable for determination of the catalytic activity of each mutant protein that carries the amino acid substitution near the C-terminal end.

We measured the catalytic activity of each recombinant ALAS2 protein with or without pyridoxal 5-phosphate (PLP). Data are summarized in Table 1. Unexpectedly, the catalytic activity of Val562Ala protein was significantly higher than that of wild-type protein ($p = 0.0046$), when the activity was measured without PLP in the assay mixture. In addition, in the presence of 200 µM PLP, Val562Ala mutant showed significantly higher activity than that of wild-type ALAS2 ($p = 0.0087$). In contrast, the catalytic activity of Met567Ile protein was lower than that of wild-type protein, irrespective of without PLP ($p = 0.0011$) or with PLP ($p = 0.0003$). It is also noteworthy that the PLP-associated increases in enzymatic activities were 86%, 44%, and 23% for wild-type, Val562Ala, and Met567Ile proteins, respectively, suggesting that Val562Ala and Met567Ile mutations decreased the responsiveness to PLP (Table 1). The lowest PLP responsiveness of Met567Ile mutant protein might account for the clinical course of the proband in case 2; that is, the anemia of this proband was improved only marginally, despite pyridoxine treatment.

Because we previously reported on the Ser568Gly mutation [15], which is also located in the C-terminal region of human ALAS2 protein, the reported data for the Ser568Gly mutation were included as a reference in Table 1. In vitro enzymatic activity of glutathione *S*-transferase (GST)–fused Ser568Gly was significantly lower than that of the GST–fused wild-type ALAS2 with or without PLP [15]. Therefore, the functional consequence of amino acid substitution at Ser568 was similar to that of Met567Ile (Table 1). In addition, the degree of PLP-mediated increase in Ser568Gly activity, indicated as "ratio with/without PLP" in Table 1, was larger than that with wild-type protein, although the possibility remains that the GST tag might have influenced the PLP responsiveness of a recombinant ALAS2 protein. We, therefore, included Ser568Gly mutant in subsequent analyses.

The higher catalytic activity of Val562Ala protein prompted us to examine the function of the C-terminal region of ALAS2 protein. We measured the enzymatic

activity of the deletion mutant that lacks the 33 amino acids at the C-terminal end (positions 555–587) of human ALAS2 (delC33 mutant), the region of which was conserved among mammalian ALAS2 proteins, including Val562. As shown in Table 1, the enzymatic activity of the delC33 mutant was higher by two times in the presence of PLP than that of wild-type ALAS2 ($p = 0.002$), whereas they showed similar enzymatic activity in the absence of PLP. These results suggest that the 33 amino acids at the C-terminal end of human ALAS2 protein might repress the enzymatic activity, probably by interfering with the access of PLP cofactor to the catalytic site.

Stability of mutant ALAS2 proteins in vivo
We were interested in studying how the Val562Ala mutation is associated with XLSA, despite higher enzymatic activity. We examined the stability of the Val562Ala mutant protein and other C-terminal mutant proteins in vivo. When human ALAS2 protein is expressed as a FLAG-tagged protein in eukaryotic cells, the precursor and mature proteins should be detected as 65.7-kDa and 60.5-kDa proteins, respectively. As shown in Figure 1B (upper panel) and Figure 2 (middle panel), FLAG-tagged wild-type ALAS2 and mutant ALAS2 proteins, except for delC33 mutant, were detected as bands at about 60 kDa, an expected size of the mature protein. These results suggest that the leader peptide at the N-terminal end was cleaved after translocation of the precursor protein into mitochondria [4]. In fact, the precursor protein was detected at an expected size, when HeLa cells were transfected with FLAG-ALAS2 expression vector, and then incubated with hemin (Supplementary Figure E2; online only, available at www.exphem.org), which is known to inhibit mitochondrial translocation of ALAS precursor protein into mitochondria [4]. Based on our experiments (Fig. 1A–C), the half-lives of wild-type and Val562Ala mature proteins in mitochondria were calculated as 7.8 hours and 5.3 hours, respectively. The half-life of the Val562Ala mutant protein (Fig. 1C) is shorter than that of wild-type ALAS2 protein (Fig. 1B). In contrast, the half-life of Met567Ile (Fig. 1D) or Ser568Gly (Fig. 1E) mutant was not measurable by our experiments because the 50% reduction of the protein level was not observed within 12 hours for these mutants. Thus, the half-lives of Met567Ile and Ser568Gly mutants were longer than 12 hours. Importantly, the amino acid substitutions in the C-terminal region influenced the stability of the mature ALAS2 protein in mitochondria in different manners. Namely, Val562Ala mutation results in destabilization of the mature protein, and either Met567Ile or Ser568Gly mutation stabilizes the mature protein in mitochondria.

In addition, we measured the half-life of delC33 mutant in HEK293-derived cells (Fig. 1F), showing that the 50% reduction was not observed within 12 hours, which was similar to Met567Ile and Ser568Gly mutants. These results

suggested that the 33 amino acids at C-terminal region of ALAS2 protein suppressed the catalytic activity in vitro, as well as protein stability in mitochondria. Our data also indicate that Val562Ala mutation might enhance the destabilization function of the C-terminal region, whereas Met567Ile and Ser568Gly mutations might enhance the suppressive function for enzymatic activity.

Enzymatic activity of each ALAS2 mutant protein in vivo
Val562Ala mutant showed higher enzymatic activity in vitro (Table 1), but it was less stable in mitochondria (Fig. 1A) compared with wild-type ALAS2. On the other hand, Met567Ile and Ser568Gly mutants showed lower enzymatic activities in vitro (Table 1), with prolonged half-lives in mitochondria (Fig. 1A). We, therefore, determined the catalytic activity of each mutant protein in vivo. For this purpose, we compared the accumulation of porphyrins in HEK293-derived cells that expressed wild-type protein or a mutant protein, as we described previously [20]; that is, the accumulation of porphyrins was evaluated by comparing the intensity of the fluorescence under ultraviolet light (Fig. 2, upper panel). The accumulation of porphyrins was detected in cells expressing wild-type ALAS2, but not in cells expressing tagged luciferase. These results indicate that FLAG-tagged ALAS2 is catalytically active in mitochondria. In contrast, the accumulation of porphyrins was decreased in cells expressing Val562Ala, Met567Ile, or Ser568Gly protein, compared to cells expressing wild-type ALAS2. Among these three missense mutations, Val562Ala mutant showed higher catalytic activity than did Met567Ile or Ser568Gly mutant (Fig. 2, upper panel). In addition, the highest porphyrin accumulation was observed in cells expressing delC33. Of note, the expression level of Val562Ala mutant protein was much lower than that of any other mutant or wild-type ALAS2, as judged by Western blot analysis (Fig. 2, middle panel), although there was no significant difference in relative expression level of each mutant ALAS2 messenger RNA (Fig. 2, lower panel). These results suggest that Val562Ala mutant protein is catalytically hyperactive but unstable in mitochondria, which is consistent in part with the higher enzymatic activity detected in vitro (Table 1) and with the short-half life in vivo (Fig. 1A).

In conclusion, Val562Ala, Met567Ile, or Ser568Gly ALAS2 has lower enzymatic activity in mitochondria compared with the activity of wild-type ALAS2. Therefore, these three mutations are categorized as a loss-of-function mutation and are responsible for sideroblastic anemia.

Discussion
It is well known that a loss-of-function mutation of the ALAS2 gene causes XLSA. In addition to the ALAS2 gene, other genes (e.g., SLC25A38 [24], GLRX5 [25], ABCB7 [26], PUS1 [27], SLC19A2 [28], and mitochondrial DNA [29]) were reported to be responsible for
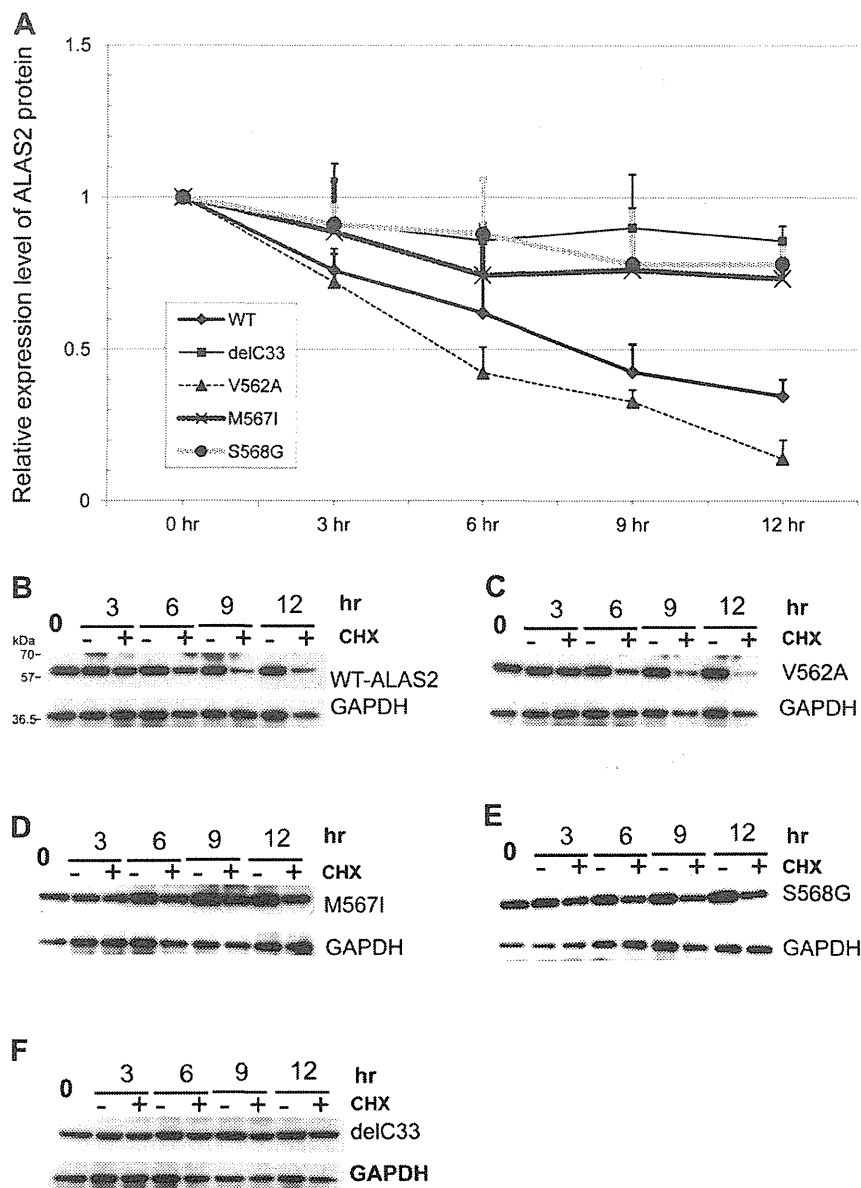
Figure 1. Effect of cycloheximide on FLAG-tagged ALAS2 protein level in eukaryotic cells. Expression of each FLAG-tagged protein was induced with tetracycline (1 μg/mL) in HEK293-derived cells for 48 hours, and then cells were treated with 10 μg/mL cycloheximide (CHX) for the indicated hours. Cells were collected and lysed in RIPA buffer, and FLAG-tagged proteins were detected by Western blot analysis (B−F). The intensity of the FLAG-tagged protein was normalized with the intensity of GAPDH for each time point. In (A), the relative intensity representing FLAG-tagged protein at 0 hours was considered to be 100%. The half-life of each protein was calculated on the basis of 50% reduction of each protein expression from the relative expression curves obtained from the samples with CHX. Average value of three independent experiments was used for preparing (A). Representative data of each ALAS2 protein are shown (B−F): (B) wild-type (WT) ALAS2; (C) Val562Ala; (D) Met567Ile; (E) Ser568Gly; and (F) delC33.

hereditary or congenital sideroblastic anemia. Among these candidate genes, mutations in ALAS2 gene are most frequently identified in patients with sideroblastic anemia [30], but characterization of each mutant ALAS2 protein was not fully performed. To the best of our knowledge, 24 of 56 mutations of the ALAS2 gene were characterized in vitro using recombinant proteins with or without a peptide-tag [9,10,14,15,20,21,31–36]. In the 11th exon

of the ALAS2 gene, Ser568Gly [15], Arg559His [17], Arg560His [16], and Arg572His [14] mutations have been reported; however, only Ser568Gly and Arg572His mutants were characterized using recombinant proteins. Concerning the Ser568Gly mutation [15], we confirmed that Ser568Gly mutation resulted in decreased enzymatic activity in vitro (about 30% of wild-type with PLP in the assay mixture), as shown in Table 1. In contrast, Ducamp et al. [14] were
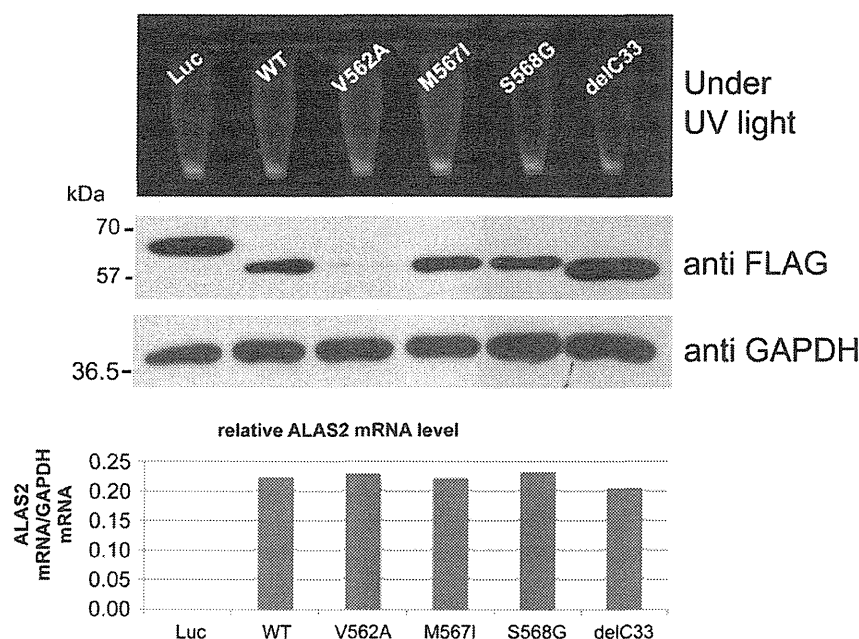
Figure 2. Evaluation of porphyrin production in cells expressing each ALAS2 mutant protein. Each FLAG-tagged ALAS2 protein or FLAG-tagged luciferase protein as a control was expressed in Flp-In T-REx 293 cells. Accumulation of porphyrins in each cell line was visualized by ultraviolet light exposure (upper panel). Expression levels of each FLAG-tagged protein and GAPDH (loading control) were detected by Western blot analysis (middle panels). Expression level of ALAS2 messenger RNA (mRNA) was measured by real-time PCR, and it was normalized with the expression level of GAPDH mRNA. Note that the data confirm the similar mRNA level of each ALAS2 protein (lower panel).

unable to determine the defect of the Arg572His mutant using an in vitro system because the mutant protein showed the enzymatic activity similar to that of wild-type ALAS2.

Measurement of enzymatic activity of every recombinant protein in vitro is one of the most useful techniques to characterize a mutant ALAS2 protein. Met567Ile mutant showed lower enzymatic activity than did wild-type protein (see Fig. 2), suggesting that this mutation causes sideroblastic anemia. In contrast, we were unable to uncover the pathogenesis of Val562Ala mutant protein using this in vitro assay system, indicating a limitation of the in vitro assay system with a bacterially expressed recombinant protein. In fact, using the in vivo system (Fig. 2), we have successfully demonstrated that the Val562Ala mutant protein showed lower porphyrin accumulation due to enzyme instability compared to wild-type ALAS2. In addition, the half-life of mature Val562Ala protein (5.3 hours) was shorter than that of wild-type ALAS2 (7.8 hours) (Fig. 1), suggesting that Val562Ala mutation altered the protein stability in mitochondria. These in vivo methods seem to be useful to characterize a mutant protein that does not show decreased enzyme activity in the in vitro assay system.

It is of particular interest that the Val562Ala and Met567Ile mutants exerted opposite effects on the enzymatic activity in vitro (Table 1) and on the protein stability in mitochondria (Fig. 1). In this connection, the deletion of 33 amino acids at C-terminal end of ALAS2 protein, the region of which contains Val562 and Met567 residues,

resulted in higher enzymatic activity in vitro and in vivo (Table 1) and stable protein with a longer half-life in mitochondria (Fig. 1). The C-terminal region has a suppressive function on enzymatic activity, as well as protein stability in mitochondria. Because this region is conserved in eukaryotic ALAS2 but is absent in prokaryotic ALAS, the suppressor domain might be involved in the functional regulation of ALAS2 in mitochondria. In fact, in the C-terminal region, two frame-shift mutations of the ALAS2 gene were reported to cause X-linked dominant protoporphyria [11], and six (including present two cases) missense mutations were identified in patients with XLSA. In addition, it was recently reported that the Tyr586Phe mutation of ALAS2 protein increased the enzymatic activity, which can contribute to the severe clinical phenotype of the patient with congenital erythropoietic porphyria [18]. These results suggest that the C-terminal region of ALAS2 functions as an intrinsic suppressor for protoporpyrin production in erythroid cells.

It is still unclear how this C-terminal region suppresses the enzymatic activity of ALAS2 protein in mitochondria. It has been reported that certain amino acids are essential for catalytic activity of mouse Alas2 [18,37–45]. However, only limited information is available concerning the role of the C-terminal region in the catalytic activity of ALAS2. To-Figueras et al. [18] performed the stoichiometric analysis of the mature ALAS2 protein to characterize Tyr586-Phe mutant, which was reported as a gain-of-function

mutation at the penultimate C-terminal amino acid of ALAS2 protein. Steady-state kinetic analyses revealed that Tyr586Phe mutant showed higher catalytic activity with greater catalytic efficiency for glycine and succinyl-CoA than those of wild-type ALAS2. In addition, these authors provided evidence that the Tyr586Phe mutant enzyme was able to form and release ALA more rapidly than wild-type enzyme. Similar mechanisms might underlie the increased activity of every C-terminal deletion mutant, including the mutant ALAS2 protein with the deletion of 19 or 21 amino acids [11] and the delC33 mutant. In addition, the delC33 mutant expressed enzymatic activity similar to wild-type ALAS2 without PLP in assay mixture, but its enzymatic activity was increased about twofold compared to the wild-type with PLP (Table 1). These results suggest that this region might be involved in efficient use of PLP or accessibility of PLP to the catalytic site.

Crystal structure analysis of homodimeric ALAS from Rhodobactor capsulatus ($ALAS_{RC}$) revealed that $ALAS_{RC}$ showed open or closed structure, which was related to the conformational change of the active site loop [17]. This active site loop consists of evolutionarily conserved structure at the C-terminal region of $ALAS_{RC}$, and seems to cover the catalytic site, which is located at the homodimer interface of ALAS protein. It should be noted that $ALAS_{Rc}$ does not contain the C-terminal region equivalent to that of mammalian ALAS2 [17]. The open conformation was observed only in the substrate-free $ALAS_{RC}$ protein, and the closed conformation was observed in $ALAS_{RC}$ protein that bound glycine and succinyl-CoA. To clarify the functional consequence of the conformational change of this active site loop, Lendrihas et al. introduced a mutation into nonconserved amino acid at this active site loop in mouse Alas2 protein and obtained several hyperactive variants [46]. Pre−steady-state kinetic analysis revealed that release of ALA from the catalytic site of the enzyme, which is coincident with opening of the active site loop [45], was accelerated in these hyperactive variants. Because the release of ALA from catalytic site is the rate-limiting step of enzymatic reaction of ALAS [47], these results suggest that the dynamic conformational change of this active site loop might control the rate of the reaction. Importantly, the accelerated release of ALA from the enzyme was also proposed in Tyr586Phe mutant [18]. It is therefore possible that the C-terminal domain of human ALAS2 protein is involved in the regulation of the conformational change of the active site loop.

In the present study, we determined the stability of ALAS2 protein in vivo, although the protein was tagged with a small peptide and expressed in HEK293-derived cells. Based on our assay condition, the half-life of human ALAS2 mature protein is 7.8 hours; however, it is not clear whether this result is comparable with that of the native ALAS2 protein in erythroid mitochondria, which has never been reported. On the other hand, this assay revealed that the stability of the Val562Ala mutant protein was decreased in mitochondria (Fig. 1), although the in vitro assay using purified recombinant protein failed to detect the unstable property of this mutant. In addition, our in vivo assay system clearly showed that the C-terminal region of 33 amino acids of human ALAS2 protein suppressed the enzymatic activity and decreased the protein stability. It is also interesting that the Val562Ala mutation and the Met567Ile mutation have opposite effects on the two functions of the C-terminal region. These results suggest that independent mechanisms might be involved in the reduction of enzymatic activity and destabilization in mitochondria. Taken together, the C-terminal region of ALAS2 protein can decrease catalytic activity by altering the open or closed structure of the catalytic site, while the post-translational modification of the C-terminal region, which is induced by a certain intracellular condition (e.g., increased or decreased oxidative stress) or by the association with other molecules, can enhance the disappearance of ALAS2 protein from mitochondria. The crystal structure of ALAS from $ALAS_{RC}$ provided critical information about the mechanisms for catalytic reaction of ALAS [45,46]. However, determination of the crystal structure of mammalian ALAS2 should await additional investigation on the function of the C-terminal region of ALAS2 protein.

Conflict of interest disclosure
No financial interest/relationships with financial interest relating to the topic of this article have been declared.

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A

B
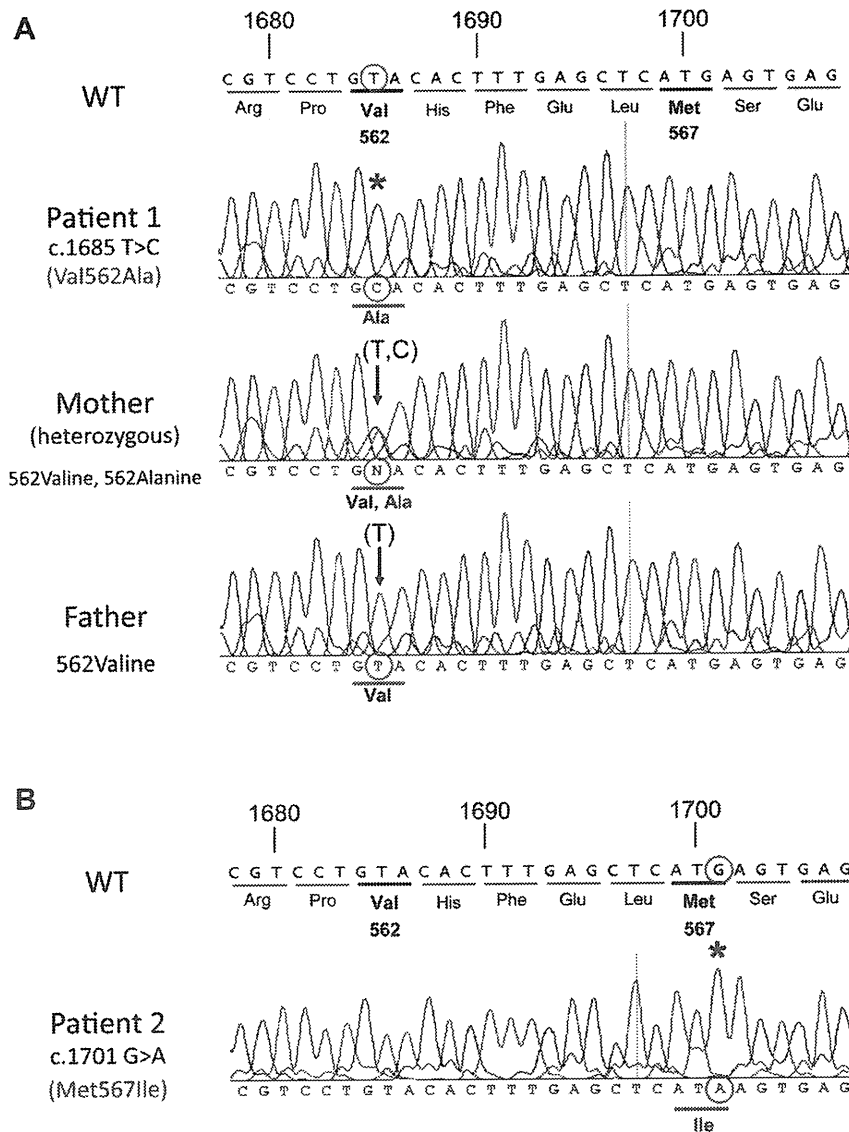
Supplementary Figure E1. Direct sequencing of 11th exon of ALAS2 gene in patients with sideroblastic anemia. Exon 11 of ALAS2 gene from each proband was amplified by PCR, and the amplicon was sequenced directly. Numbers shown at top indicate the positions of cDNA sequence, which is started from the first nucleotide of the ATG-translation initiation codon. Second and third lines indicate wild-type DNA sequence and amino acid sequence, respectively. Identified mutations are indicated with asterisks, and the expected amino acid substitution is shown under each mutation. (A) The c.1685T > C mutation of ALAS2 gene in case 1. The heterozygous condition of proband's mother and the wild-type allele of proband's father are shown. (B) The c.1701G > A mutation of ALAS2 gene in case 2.
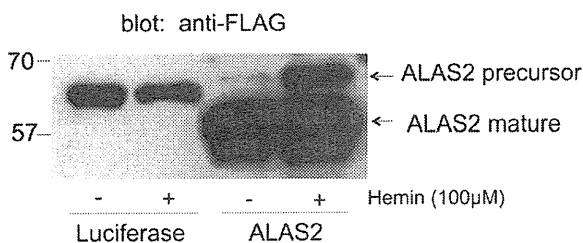
Supplementary Figure E2. Transient expression of FLAG-ALAS2 and FLAG-Luciferase in HeLa cells. HeLa human cervical cancer cells were transfected with FLAG-ALAS2 or FLAG-luciferase expression vector, then treated with 100 μM hemin. Cell lysates were subjected to the Western blot analysis with anti-FLAG antibody. Shown are the representative data.

Matched sibling donor stem cell transplantation for Fanconi anemia patients with T-cell somatic mosaicism

Yabe M, Shimizu T, Morimoto T, Koike T, Takakura H, Tsukamoto H, Muroi K, Oshima K, Asami K, Takata M, Yamashita T, Kato S, Yabe H. Matched sibling donor stem cell transplantation for Fanconi anemia patients with T-cell somatic mosaicism.

Abstract: SCT from HLA-identical sibling donors is generally associated with an excellent survival in FA patients if performed prior to the development of MDS or leukemia. However, the optimal conditioning regimen has not been defined. We report here our experience with 15 Japanese FA patients who underwent HLA-matched sibling donor SCT. The aim of this study is to compare radiation-based conditioning to Flu-based conditioning for FA patients in a Japanese population where the T-cell somatic mosaicism is higher than in the Caucasian population. Eight patients (a-group) received a radiation-based conditioning (500–600 cGy of thoracoabdominal/TBI) with CY dose modification (20–120 mg/kg), and ATG; two patients exhibited rejection. Seven patients (b-group) received CY (40 mg/kg), 150–180 mg/m^2 of Flu, and ATG. Durable engraftment was demonstrated in all patients. In FA patients, Flu-based conditioning may allow stable engraftment in matched sibling donor transplantation without radiation, even in patients with T-cell somatic mosaicism.

Miharu Yabe[1], Takashi Shimizu[2], Tsuyoshi Morimoto[2], Takashi Koike[2], Hiromitsu Takakura[2], Hideo Tsukamoto[3], Kazuo Muroi[4], Koichi Oshima[5], Keiko Asami[6], Minoru Takata[7], Takayuki Yamashita[8], Shunichi Kato[1] and Hiromasa Yabe[1]

[1]Department of Cell Transplantation, [2]Department of Pediatrics, and [3]Teaching and Research Support Center, Tokai University Hospital, Kanagawa, [4]Division of Cell Transplantation and Transfusion, Jichi Medical School, Tochigi, [5]Division of Hematology and Oncology, Saitama Children's Medical Center, Saitama, [6]Department of Pediatrics, Niigata Cancer Center, Niigata, [7]Laboratory of DNA Damage Signaling, Department of Late Effect Studies, Radiation Biology Center, Kyoto University, Kyoto, [8]Laboratory of Molecular Genetics, The Institute for Molecular and Cellular Regulation, Gunma University, Gunma, Japan

Key words: Fanconi anemia – stem cell transplantation – HLA-matched sibling donors – fludarabine – T-cell somatic mosaicism

Miharu Yabe, MD, Department of Cell Transplantation, Tokai University Hospital, Shimokasuya 143, Isehara, Kanagawa 259-1193, Japan
Tel.: 81 463 93 1121
Fax: 81 463 93 8607
E-mail: miharu@is.icc.u-tokai.ac.jp

Somatic mosaicism, the presence of non-FA cells among FA hematopoietic cells, has been considered a risk factor for engraftment in SCT

Abbreviations: ALG, antilymphocyte globulin; ATG, antithymocyte globulin; BMT, bone marrow transplantation; CsA, cyclosporine A; CY, cyclophosphamide; DEB, diepoxybutane; FA, Fanconi anemia; Flu, fludarabine; GVHD, graft-vs.-host disease; HLA, human leukocyte antigen; INFA, International Fanconi Anemia Registry; MDS, myelodysplastic syndrome; MMC, mitomycin-C; MTX, methotrexate; RA, refractory anemia; RRT, regimen-related toxicity; SCT, stem cell transplantation; TAI, thoracoabdominal irradiation; TBI, total body irradiation.

from alternative donors, because DEB-resistant T-cells may increase the risk of graft rejection (1). Wagner et al. (2) reported that engraftment was poorer in unrelated donor recipients with T-cell somatic mosaicism not treated with a Flu-containing regimen. We reported that there is a high frequency of T-cell somatic mosaicism in Japanese FA patients (3). The current study presents the results of matched sibling donor SCT in 15 FA patients undergoing two types of conditioning regimens: radiation-based conditioning with CY dose modification and Flu-based regimen without radiation.

Patients and methods

Patients and donor selection

Between 1987 and 2008, 15 patients with FA received 17 SCTs from their HLA-matched sibling donors. The diagnosis of FA was confirmed by chromosomal breakages induced by MMC or 0.1 μg/mL DEB. The chromosomal fragility test for CY metabolites was performed using the serum obtained from other CY-treated, non-FA SCT patients with severe aplastic anemia as previously described (4). We chose the concentration of 0.4 μg/mL for CY metabolites in chromosomal fragility testing of FA patients because it induces multiple chromosomal breaks in FA patients while having little clastogenic effect on normal cells (4). Peripheral lymphocytes from 78 FA patients underwent both CY metabolite and DEB tests in our laboratory. Patients with 50% or more cells insensitive to treatment with 0.1 μg/mL DEB and/or 0.4 μg/mL CY metabolite were classified as high-mosaic in this study for comparison with patients in the IFAR (5). Patient characteristics are shown in Table 1. Age at transplant ranged from 5 to 24 yr. Twelve patients had severe aplastic anemia, and three had RA. Determination of complementation groups was informative in 11 patients, of whom six were placed in group A and five in group C. Of our 15 patients, seven had received androgen therapy, and all 15 were transfusion-dependent at the time of SCT. All 15 donors (14 bone marrow, one cord blood for patient 8) had negative results of DEB/MMC test prior to transplant and were matched (at HLA loci A, B, and DR) to HLA-identical siblings. Harvested marrow was not manipulated.

Conditioning regimen and GVHD prophylaxis

The conditioning regimen of eight patients of a-group (Nos. 1–8) consisted of the following: modified CY (20–120 mg/kg), either TAI (500–600 cGy) or TBI, and ATG or ALG.

We provisionally reduced the dose of CY according to the increase of chromosomal breaks case by case, although we set a minimum dose of 20 mg/kg.

Since 2000, Flu – an antimetabolite and immunosuppressive agent – has been used as a part of conditioning. The regimen of seven patients of b-group (Nos. 9–15) included CY (40 mg/kg), Flu (150–180 mg/m^2), and ATG, without radiation. GVHD prophylaxis was carried out using CsA; short-term MTX (15 mg/m^2 on day 1; 10 mg/m^2 on days 3, 5, and 11) administration was utilized in patients older than 10 yr.

Analysis of chimerism

Engraftment on the bone marrow was assayed using short tandem repeats analysis, XY chromosomal analysis, or fluorescence in situ hybridization with XY chromosome-specific probes.

The Tokai University Hospital institutional review board approved the collection and reporting of these data.

Results

Chromosomal fragility test

Table 2 shows a summary of the results of cytogenetic testing of 78 FA patients; there was a linear correlation between the percentage of aberrant metaphases in lymphocytes treated with CY metabolites and the percentage in lymphocytes treated with DEB ($r = 0.868$) (Fig. 1). Among the 12 patients tested for DEB, four were shown to be high-mosaic: two in a-group and two in b-group (Table 1). In the CY metabolite test, eight of 15 patients (four in each group) were shown to have high-mosaic.

Table 1. Patient characteristics; (a) radiation-based conditioning with CY dose modification group; (b) fludarabine-based conditioning without radiation group

No.	Age at SCT (yr)	Sex	Status at SCT (% blasts)	Clonal abnormality	Complementation group	CY test	CY mosaic (%)	DEB mosaic (%)	Prior therapy	No. of prior blood transfusions
(a)										
1	8	F	SAA (0)	No	A	0.16	91.0	56.0	PSL, Androgen	>20
2	6	M	SAA (0)	No	C	0.43	73.2	NT	None	1–20
3	14	M	SAA (0)	No	C	0.44	79.4	NT	Androgen	1–20
4	11	M	SAA (0)	No	Unknown	0.23	77.0	NT	PSL	1–20
5	5	M	SAA (0)	No	C	1.12	47.0	34.0	GCSF, Epo, CsA	1–20
6	10	M	SAA (0)	No	C	2.85	20.0	13.3	PSL, Androgen	1–20
7	24	F	RA (<5)	del(7)(p12)	Unknown	2.66	1.0	0	PSL, CsA, Androgen	1–20
8	5	F	RA (<5)	No	C	0.96	49.5	56.0	PSL, Androgen	1–20
(b)										
9	8	F	SAA (0)	No	A	0.52	58.0	54.0	None	1–20
10	15	F	SAA (0)	No	Unknown	0.81	50.0	8.0	None	1–20
11	6	M	SAA (0)	No	Unknown	2.91	13.0	4.9	Androgen	1–20
12	6	M	SAA (0)	No	A	1.05	27.0	17.0	Androgen	1–20
13	1	M	RA (<5)	Add(2)(q33)	A	0.20	82.0	63.0	None	>20
14	9	F	SAA (0)	No	A	0.61	66.0	16.0	None	1–20
15	4	F	SAA (0)	No	A	2.60	15.0	3.0	None	>20

F, female; M, male; SAA, severe aplastic anemia; CY test, cyclophosphamide metabolites-induced (0.4 μg/mL) test, with results listed as mean number of chromosome breaks per cell; CY mosaic, percentage of cells with 0.4 μg/mL cyclophosphamide metabolites-insensitivity; DEB mosaic, percentage of cells with 0.1 μg/mL diepoxybutane insensitivity; NT, not tested; PSL, prednisolone; GCSF, granulocyte colony-stimulating factor; Epo, erythropoietin.

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Table 2. Chromosome fragility test performed with DEB and CY metabolites in 78 FA patients

Agent	Breaks/cell, n	Aberrant cells, %	Aberrations/aberrant cell, n
Spontaneous	0.0 6 ± 0.09 (0–0.49)	7.93 ± 10.63 (0–66)	1.04 ± 0.16 (1–2)
DEB (0.1 μg/mL)	3.10 ± 2.67 (0.03–12.0)	68.63 ± 27.64 (2–100)	3.85 ± 2.37 (1–12)
CY metabolites (0.4 μg/mL)	1.38 ± 1.16 (0–5.57)	54.91 ± 24.61 (1–100)	2.20 ± 1.02 (1–5.93)

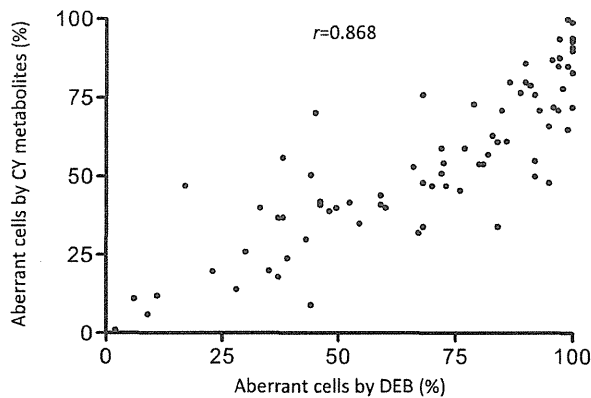DEB, diepoxybutane; CY, cyclophosphamide; FA, Fanconi anemia.

Fig. 1. Linear correlation between the percentage of aberrant metaphases in lymphocytes treated with 0.4 μg/mL CY metabolites and the percentage in lymphocytes treated with 0.1 μg/mL-DEB.

Engraftment and chimerism

The transplantation characteristics and the follow-up characteristics after transplantation are summarized in Table 3 and 4, respectively. Seven of eight patients in a-group engrafted. Patient 1, who was high-mosaic with 56% DEB-insensitive and 91% CY metabolites-insensitive lymphocytes, did not engraft; this patient received an infusion of marrow cells from another HLA-matched sibling after a conditioning regimen of TBI (8 Gy) + CY (150 mg/kg) + ALG, and engrafted. Patient 7 achieved successful engraftment after receiving 20 ng/kg of CY without any RRT. Although she was not high-mosaic, she developed late graft rejection on day 205 after pure red cell aplasia. Chromosomal analysis of peripheral blood cultured with phytohemagglutinin and that of bone marrow showed mixed chimerism. She received a second BMT from the same brother on day 240 after TBI (7.5 Gy) + CY (60 mg/kg) + ALG, and successfully engrafted.

Successful engraftment was achieved in all seven patients in b-group, independent of the proportion of DEB- or CY-insensitive cells

Table 3. Transplantation characteristics; (a) radiation-based conditioning with CY dose modification group; (b) fludarabine-based conditioning without radiation group

No.	Conditioning	TNC/kg (×10⁸)	Engraftment Day ANC >0.5 × 10⁹/L	Day platelet >50 × 10⁹/L	Organ toxicity (Bearman) Grade I/II	GVHD Acute	Chronic
(a)							
1-1	CY(40)* + TAI(6) + ALG	4.7	Rejection	Rejection	No	NE	NE
1-2	CY(150) + TBI(8) + ALG	4.5	21	74	Stomatitis, mucositis	0	Lim
2	CY(40) + TAI(6) + ALG	4.5	27	22	Stomatitis	0	–
3	CY(45) + TAI(6) + ALG	2.5	12	27	Stomatitis	0	Lim
4	CY(120) + TAI(6) + ALG	1.7	13	26	No	0	–
5	CY(20) + TAI(6)	6.6	16	21	No	0	–
6	CY(20) + TAI(6) + ATG	3.1	17	33	No	0	–
7-1	CY(20) + TAI(5) + ALG	≥2.0	14/No	16/No	No	0	–
7-2	CY(60) + TBI(7.5) + ALG	≥2.0	15	Unknown/Yes	Stomatitis, liver	II	Ext
8	CY(40) + TBI(6) + ATG	0.21† CB	15	38	Stomatitis	0	–
(b)							
9	CY(40) + Flu(180) + ATG	6.2	10	12	No	0	–
10	CY(40) + Flu(150) + ATG	5.2	14	23	Stomatitis, liver	0	–
11	CY(40) + Flu(150) + ATG	3.5	14	31	Stomatitis	0	–
12	CY(40) + Flu(150) + ATG	3.3	11	24	No	0	–
13	CY(40) + Flu(150) + ALG	7.8	9	17	Stomatitis	0	–
14	CY(40) + Flu(150) + ATG	4.1	11	17	Stomatitis	0	–
15	CY(40) + Flu(150) + ATG	3.9	12	24	No	0	–

TNC, total nucleated cells; NE, not evaluable.
*The number in parentheses indicates the dose, and units are Gy for TAI and TBI, mg/m² for Flu, and mg/kg for CY.
†Post-thawing cell dose of cord blood.

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Table 4. Follow-up characteristics after transplantation; (a) radiation-based conditioning with CY dose modification group; (b) fludarabine-based conditioning without radiation group

| No. | Chimerism status (% donor cells) | | Complication (months after SCT) | Outcome (months after SCT) |
	At the first time (source/days after SCT)	At the last time (source/months after SCT)		
(a)				
1-1	NE	NE	Hepatitis B and C	Rejection. 2nd SCT
1-2	100 (BM/42)	NT	Ovarian dysfunction Hepatic carcinoma (145)	Dead/hepatic carcinoma (147)
2	100 (BM/41)	100 (PB/280)	Prediabetic state	Alive (280)
3	NT	NT	Tongue carcinoma (102)	Dead/tongue carcinoma (114)
4	NT	NT	No	Alive (250)
5	NT	NT	No	Alive (220)
6	100 (BM/14)	100 (BM/2)	No	Dead/accident (154)
7-1	100 (BM/15)	50 (PB/3)	Ovarian dysfunction	PRCA/late rejection, 2nd SCT (eight months)
7-2	100 (BM/15)	100 (PB/60)	Esophagus carcinoma (138) Tongue carcinoma (177)	Alive with cancer disease (234)
8	100 (PB/15)	100 (PB/178)	Pheochromocytoma (147) Prediabetic state	Alive (178)
(b)				
9	100 (BM/12)	100 (PB/105)	No	Alive (129)
10	100 (BM/32)	100 (PB/81)	No	Alive (117)
11	100 (BM/14)	100 (PB/60)	No	Alive (86)
12	98.8 (BM/14)	100 (PB/36)	No	Alive (48)
13	100 (BM/28)	100 (PB/17)	No	Alive (46)*
14	100 (BM/14)	100 (PB/46)	No	Alive (46)
15	100 (BM/14)	95 (PB/41)	No	Alive (41)

NE, not evaluable; BM, bone marrow; NT, not tested; PB, peripheral blood; PRCA, pure red cell aplasia.
*This case was reported by Oshima et al. (21).

(ranges, 3–63% and 13–82%, respectively). The time to an absolute neutrophil count (ANC) $> 0.5 \times 10^9/L$ was 9–14 days, and to a platelet count of $50 \times 10^9/L$ was 12–31 days. In patients of b-group, chimerism of their BM mononuclear cells in the early stages after SCT was 98.8–100% of donor type. Although two of these seven patients showed transient mixed chimerism (patient 14 and 15) in the peripheral blood mononuclear cells during the first year after SCT, all patients achieved > 95% donor chimerism.

Toxicity

The grade of toxicity was low both in two groups. None of the patients showed grade III/VI RRT (Bearman's criteria) at any evaluation point.

GVHD

Only one patient (patient 7) developed grade II acute GVHD after the second transplant, and chronic GVHD was observed in three patients who developed malignancies as late effects (patient 1, 3 and 7). No patients of b-group had acute and/or chronic GVHD.

Outcome

In patients of a-group, hepatocarcinoma and tongue/esophagus carcinoma were observed in patients 1 and 7, at 12 and 10/14 yr after BMT, respectively. Tongue carcinoma and pheochromocytoma were also observed in patients three and eight, at nine and 12 yr after BMT, respectively. Causes of death in a-group were solid cancer in two of eight, and one died of accident. Two patients suffered from ovarian dysfunction (patient 1 and 7), and two patients are in prediabetic state (patient 2 and 8). None of the b-group required a second SCT (median follow-up, 48 months; range, 41–129 months); all patients are alive with a Lansky/Karnofsky score of 100%, and there are no late side effects such as ovarian failure or other endocrinopathy.

Discussion

Graft rejection, RRT, and severe acute GVHD have been the major causes of SCT failure in FA patients. However, in FA patients, SCT from an HLA-identical sibling donor is generally associated with an excellent outcome when performed before leukemic transformation. The approach used by Gluckman et al. (6), including low-dose

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CY (20–40 mg/kg) + TAI/TBI (400–450 cGy), has been the standard SCT conditioning regimen. This conditioning regimen provided good results, with >80% survival at 3–10 yr (7, 8). Non-radiation regimens have been increasingly used for FA patients to reduce the late effects associated with radiation, such as endocrinopathies, infertility, and cataracts.

Bonfim et al. (9) reported using only CY (60 mg/kg) in 43 patients from matched-related SCT donors; Ayas et al. (10) also reported using a CY (60 mg/kg) + ATG regimen without radiation in 34 patients with matched-related donor SCT. Overall survival rate in these studies were 93% (median follow-up, 3.7 yr) and 96.9% (median follow-up, 33.7 months), respectively. However, MacMillan et al. (1) observed a high rate of graft failure in FA patients receiving unrelated donor transplants with T-cell somatic mosaicism, suggesting that the presence of DEB-insensitive T-cells increased the risk of graft rejection.

We showed that there was a linear correlation between the percentage of aberrant metaphases in lymphocytes treated with CY metabolites and those treated with DEB (Fig. 1). On the basis of these data, we suggested that DEB-insensitive cells are also CY-insensitive cells. Therefore, incomplete ablation of DEB-resistant host lymphocytes might increase the risk of graft failure. If patients with 50% or more DEB-insensitive cells are classified as high-mosaic, only 10% of FA patients have been reported to exhibit >50% insensitive cells in IFAR patients (5); however, in our study, among the 78 patients, 24 were high-mosaic (30.8%). A conditioning regimen that exhibits strong cytotoxic activity against lymphocytes and minimized exposure to DNA cross-linking agents may be necessary for Japanese FA patients because the T-cell somatic mosaicism in Japanese population is higher than the Caucasian population. The patients in a-group received high-dose therapy, particularly those who received a second transplant. This might explain the high incidence of cancer. Furthermore, the two patients who developed tongue carcinoma in a-group had oral chronic GVHD. There was a significant association of the oral squamous cell carcinoma with chronic GVHD (11, 12).

In recent years, Flu-containing conditioning regimens for FA have become more popular and have been successfully employed, especially in SCT from alternative donors (2, 13, 14). Flu is an antimetabolite and immunosuppressive agent that is not a DNA cross-linking agent. The first FA patient with leukemic transformation successfully treated by matched sibling BMT following a Flu-based conditioning regimen was reported in 1997 (15). Flu is an attractive and tolerable agent for FA because it is not an alkylating agent and has an antileukemic effect. Furthermore, omission of irradiation from a conditioning regimen has been considered to reduce the late effects. Tan et al. (16) reported that 11 patients with 0–20% DEB-insensitive cells had received a conditioning regimen of CY (20 mg/kg) + Flu (175 mg/m^2) + ATG without irradiation, followed by an infusion of HLA-genotypically identical T-cell-depleted bone marrow or cord blood. Neutrophil engraftment was observed in all patients, but secondary graft failure was observed in one patient. No patients experienced severe RRT or either acute or chronic GVHD, and nine are alive and well at a median follow-up of 2.9 yr. Ertem et al. (17), who used a similar regimen (CY 20 mg/kg + Flu 150 mg/m^2 + ATG), reported successful engraftment in 6 FA patients. Stepensky et al. (18) also reported that a combination of Flu with ATG and low-dose CY without radiation was safe and demonstrated low rejection rates when compared with alternative regimens in patients with FA. After 2000, we selected Flu, a consistent, reduced dose of CY 40 mg/kg, and ATG without radiation as conditioning for HLA-matched sibling donor SCT, and successful engraftment was achieved in all seven patients with stable chimerism, independent of the T-cell somatic mosaicism. They are all disease free and in good clinical condition without any late side effects.

Age at SCT (>10 yr) is also a risk factor of acute GVHD (19). To prevent moderate-to-severe acute GVHD, we have used the combination of CsA plus short-term MTX in patients older than 10 yr in matched sibling donor SCT and used tacrolimus plus short-term MTX after alternative donor transplant (13). No patients had severe MTX toxicity, and none of them died of acute GVHD in either a- and b-group. Bonfim et al. (9) have also used CsA plus short-term MTX in HLA-matched related donor SCT in 43 FA patients. They found a very low incidence of acute GVHD and suggested that less regimen-related tissue damage enabled the delivery of all four scheduled MTX doses in the majority of patients; MTX dosing was previously shown to be important in controlling the incidence of GVHD (20). These combinations of GVHD prophylaxis including MTX considerably decreased the severe acute GVHD for FA patients, which could have varied in accordance with ethnic differences.

Our study indicates that a Flu-based regimen without radiation enabled successful engraftment

in HLA-matched sibling donor SCT even in FA patients with evidence of T-cell somatic mosaicism. It is very difficult to compare the outcome of two different regimens as there are two second transplant in a-group and the major differences between the follow-up times of two groups. Long-term follow-up and larger studies are warranted to confirm the high engraftment rates and reduction of post-transplant malignancies.

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Disclosure

The authors declare no competing financial interests.

Author contributions

M. Yabe, H. Yabe: concept/design; M. Yabe, T. Shimizu, T. Morimoto, T. Koike, H. Takakura, H. Tsukamoto, K. Muroi, K. Asami, K. Oshima, M. Takata, T. Yamashita: data analysis/interpretation; S. Kato, H. Yabe: approval of the article.

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Ⅲ．診断と治療

7．先天性骨髄不全症候群

小島　勢二[1)]　矢部みはる[2)]

要　旨

　先天性骨髄不全症候群は，造血細胞の分化，増殖が先天的に障害され，血球減少をきたす疾患の総称で，Fanconi貧血のように汎血球減少を呈する疾患から，Diamond-Blackfan貧血のように単一系統の血球の減少をきたす疾患までを含む．特徴的な外表奇形がみられることから，従来は臨床診断がおこなわれてきたが，分子生物学の進歩により責任遺伝子の発見が続き，多くの疾患において，遺伝子診断も可能となっている．

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Key words　責任遺伝子，Fanconi貧血，先天性角化不全症，造血幹細胞移植

はじめに

　先天性骨髄不全症候群は，造血細胞の分化・増殖が先天的に障害され，血球減少をきたす疾患の総称である．血球減少に加え，特徴的な外表奇形や所見を伴うことから，従来は臨床診断がなされてきたが，近年になって責任遺伝子が，相次いで同定され，遺伝子診断にもとづく正確な診断が可能になってきた．しかし，今日でも，臨床診断がついた症例のうち責任遺伝子の変異が発見されるのは，半数にすぎない．

　汎血球減少をきたす先天性骨髄不全症候群にはFanconi貧血（FA），dyskeratosis congenita（DC），Shwachman-Diamond症候群（SD）が含まれる．また，単一血球系統に限定される血球減少症には，Diamond-Blackfan貧血（DBA），遺伝性鉄芽球性貧血，congenital dyserythropoietic anemia（CDA），先天性重症好中球減少症（SCN），先天性無巨核球性血小板減少症（CAMT）などがある．多くは，生下時あるいは年少児期に発症するが，一部のFAやDC患者は成人になって初めて診断される例もある．表1には，現在判明しているこれらの疾患の責任遺伝子をしめす．

　先天性骨髄不全症候群のうち，最も頻度が高いFAでもわが国における発症頻度は年間わずか5～7人であり，その他の疾患はさらに少数である．血球減少をきたし，先天性骨髄不全症候群と鑑別を必要とする疾患には，再生不良性貧血や骨髄異形成症候群（MDS）などの後天性疾患も含まれるが，その発症頻度は低く，主要な施設においても新規の患者数は年間1～2例にすぎ

トピックス

表 1. 先天性骨髄不全症候群

疾患		責任遺伝子	遺伝形式	推定される遺伝子の機能
汎血球減少症	Fanconi anemia	FANCA	AR	DNA 障害の修復
		FANCB	XR	
		FANCC	AR	
		FANCD1	AR	
		FANCD2	AR	
		FANCE	AR	
		FANCF	AR	
		FANCG	AR	
		FANCI	AR	
		FANCJ	AR	
		FANCL	AR	
		FANCM	AR	
		FANCN	AR	
	Shwachman-Diamond syndrome	SBDS	AR	リボゾーム蛋白の成熟，紡錘糸の安定化
	Dyskeratosis congenita	DKC1	XR	リボゾーム蛋白の成熟，テロメアの複製
		TERC	AD	テロメアの複製・保護
		TERT	AD, AR	
		TINF2	AD	
		NHP2	AR	
		NOP10	AR	
		TCAB1	AR	
単一系統の血球減少症	赤芽球系 Diamond-Blackfan anemia	RPS19	AD	リボゾーム蛋白の成熟
		RPS24	AD	
		RPS17	AD	
		RPS7	AD	
		RPS10	AD	
		RPS26	AD	
		RPL5	AD	
		RPL11	AD	
		RPL35A	AD	
	Hereditary sideroblastic anemia	ALAS2	XR	ヘムの合成
		GLRX5	AR	鉄/硫黄タンパク質の合成
		SLC25A38	AR	
	Hereditary sideroblastic anemia with ataxia	ABCB7	XR	ヘム輸送の担体
	Congenital dyserythropoietic anemia（type I）	CDAN1	AD	
	（type II）	SEC23B	AR	
	（unclassified）	KLF1		
	骨髄球系 Severe congenital neutropenia	HAX1	AR	好中球のアポトーシス抑制
		ELA2	AD	G-CSF，G-CSF受容体の分解
		GFI1	AD	ELA2 の上流にあり，転写を調節
		CSF3R	AR	G-CSF受容体
		WAS	XR	actinの重合，細胞骨格の制御
		G6PC3	AR	グルコース6リン酸の脱リン酸化
	Cyclic neutropenia	ELA2	AD	G-CSF，G-CSFRの分解
	血小板系 Congenital amegakaryocytic thrombocy-topenia	MPL	AR	TPO受容体

AR；常染色体劣性，AD；常染色体優性，XR；X連鎖劣性