特集 知っておくべき『肝疾患と皮膚病変』—ウイルス性肝炎治療を中心に

topics 3 肝障害と晩発性皮膚ポルフィリン症

例などでは肝細胞癌の有無を精査する必要がある．fPCTは浸透率が低く，*UROD*遺伝子に変異をもっているとしても，発症する割合は10%程度である．このことは家族歴のないsPCTと思われる症例であっても*UROD*遺伝子変異をもっている可能性を示唆している．したがって，sPCTが疑われる場合は，遺伝子診断を行ったほうがよいと思われる．

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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

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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

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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 13,200*g* 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

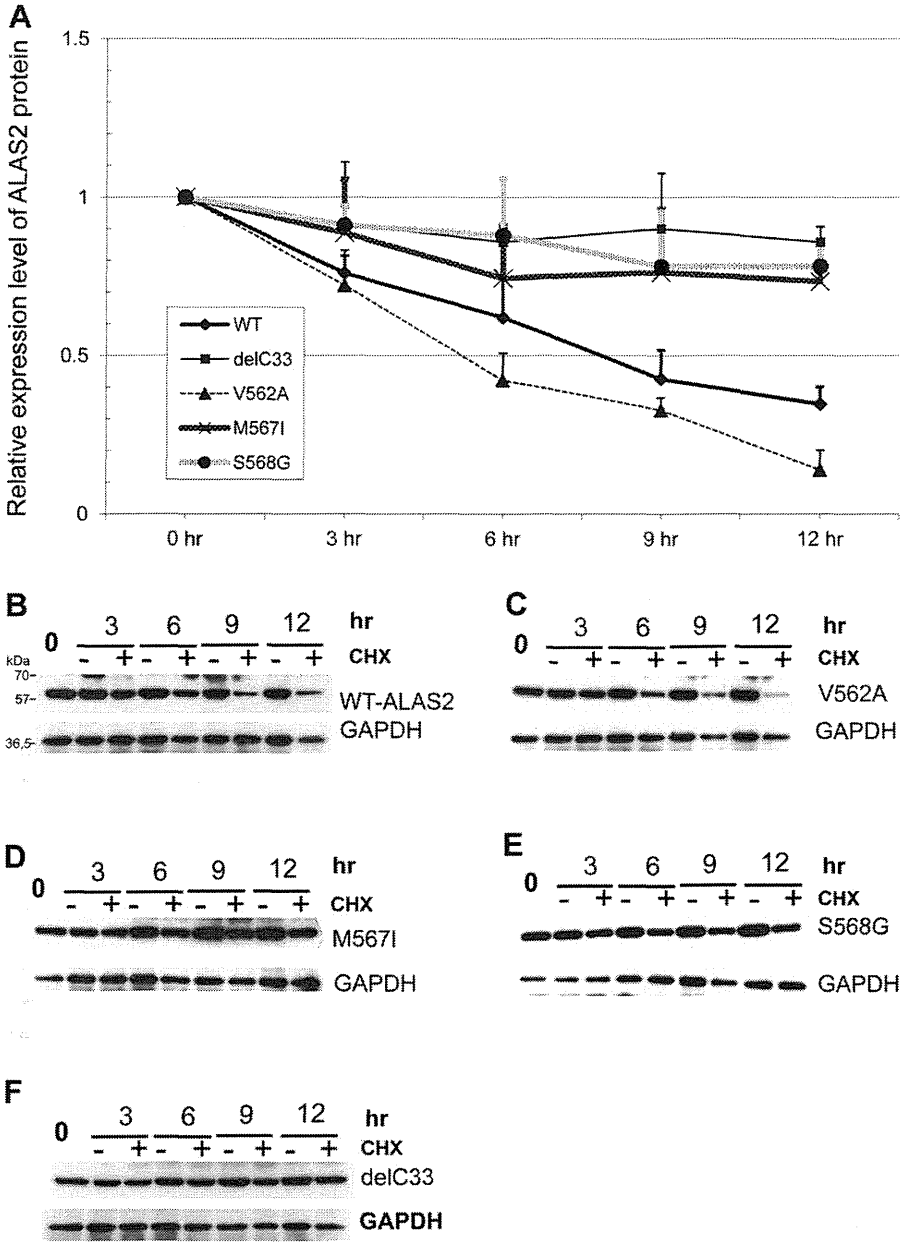

Figure 1. Effect of cycloheximide on FLAG-tagged ALAS2 protein level in eukaryotic cells. Expression of each FLAG-tagged protein was induced with tetracycline (1 μg/mL) in HEK293-derived cells for 48 hours, and then cells were treated with 10 μg/mL cycloheximide (CHX) for the indicated hours. Cells were 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

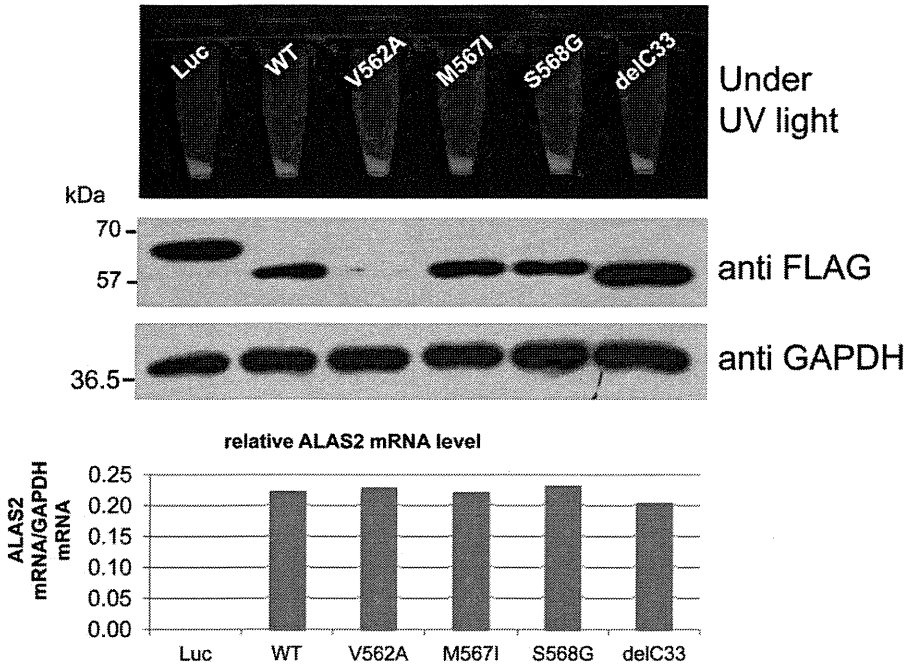

Figure 2. Evaluation of porphyrin production in cells expressing each ALAS2 mutant protein. Each FLAG-tagged ALAS2 protein or FLAG-tagged luciferase protein as a control was expressed in Flp-In T-REx 293 cells. Accumulation of porphyrins in each cell line was visualized by ultraviolet light exposure (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 evolutionally 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.

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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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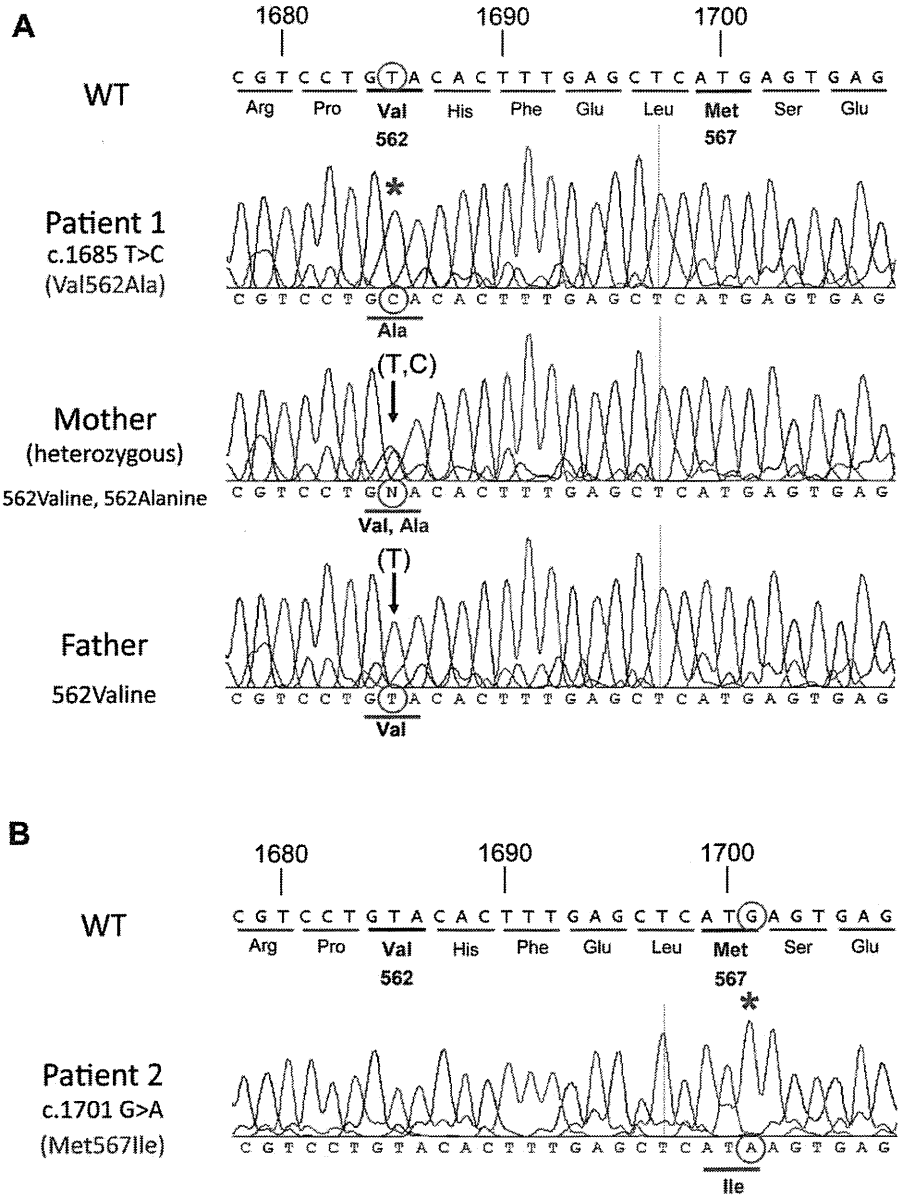
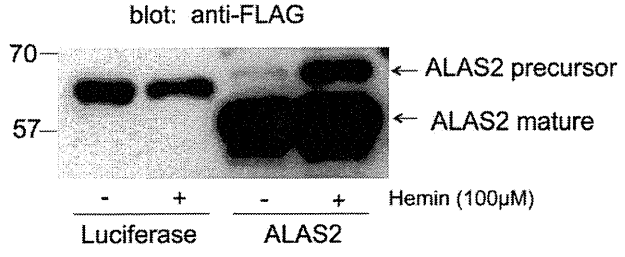


Supplementary Figure E1. Direct sequencing of 11th exon of ALAS2 gene in patients with sideroblastic anemia. Exon 11 of ALAS2 gene from each proband was amplified by PCR, and the amplicon was sequenced directly. Numbers shown at top indicate the positions of cDNA sequence, which is started from the first 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.

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ORIGINAL ARTICLE

Clinical and genetic characteristics of congenital sideroblastic anemia: comparison with myelodysplastic syndrome with ring sideroblast (MDS-RS)

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Abstract Sideroblastic anemia is characterized by anemia with the emergence of ring sideroblasts in the bone marrow. There are two forms of sideroblastic anemia, i.e., congenital sideroblastic anemia (CSA) and acquired sideroblastic anemia. In order to clarify the pathophysiology of sideroblastic anemia, a nationwide survey consisting of clinical and molecular genetic analysis was performed in Japan. As of January 31, 2012, data of 137 cases of sideroblastic anemia, including 72 cases of myelodysplastic syndrome (MDS)–refractory cytopenia with multilineage dysplasia (RCMD), 47 cases of MDS–refractory anemia with ring sideroblasts (RARS), and 18 cases of CSA, have been collected. Hemoglobin and MCV level in CSA are significantly lower than those of MDS, whereas serum iron level in CSA is significantly higher than those of MDS. Of 14 CSA for which DNA was available for genetic analysis, 10 cases were diagnosed as X-linked sideroblastic anemia due to *ALAS2* gene mutation. The mutation of *SF3B1* gene, which was frequently mutated in MDS-RS, was not detected in CSA patients. Together with the difference of clinical data, it is

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suggested that genetic background, which is responsible for the development of CSA, is different from that of MDS-RS.

Keywords Congenital sideroblastic anemia · Myelodysplastic syndrome · ALAS2

Introduction

Sideroblastic anemia is characterized by anemia with the emergence of ring sideroblasts in the bone marrow. Ring sideroblasts are formed by the irregular accumulation of iron in mitochondria. There are two forms of sideroblastic anemia i.e., congenital sideroblastic anemia (CSA) and acquired sideroblastic anemia. Most of acquired sideroblastic anemia cases were included in myelodysplastic syndrome (MDS). To date, mutations of genes involved in heme biosynthesis, Fe–S cluster biogenesis, or the biology of mitochondria have been reported in CSA [1–5]. Impaired function of these genes is speculated to result in disutilization of iron, leading to accumulation of iron in mitochondria. Acquired sideroblastic anemia in MDS is categorized either as refractory cytopenia with multilineage dysplasia (RCMD) or refractory anemia with ring sideroblasts (RARS) depending on the level of dysplasia. In contrast CSA, mechanism of forming ring sideroblasts in MDS is not clarified, although it was recently suggested that the mutations of splicing pathway are involved in the pathogenesis of MDS [6]. It is possible that there is a common mechanism between CSA and MDS; however, mutations in genes, which are responsible for development of the CSA, have not been identified in MDS.

The most common form of CSA is X-linked sideroblastic anemia (XLSA), which is caused by mutation of erythroid-specific 5-aminolevulinate synthase (*ALAS2*), the first enzyme of heme synthesis in erythroid cells [7–10]. More than half of the patients with XLSA respond to the administration of pyridoxine [vitamin B6 (Vit.B6)], or pyridoxal 5-phosphate (PLP), which is the coenzyme of *ALAS2* [11]. In XLSA, adult onset cases have been reported [12, 13]; therefore, it is possible that some cases of CSA may be misdiagnosed as MDS, especially RARS. However, the clinical and pathological features of congenital and acquired sideroblastic anemia have not been fully clarified because there have been no comprehensive studies, including clinical and genetic analyses, focusing on sideroblastic anemia.

Here, we performed a nationwide survey of sideroblastic anemia in Japan to investigate the epidemiology and pathogenesis of this disease. The difference of clinical data and results of genetic analysis suggest that genetic background, which is responsible for the development of CSA, is distinct from that of MDS-RS.

Materials and methods

Data acquisition

This study consisted of three investigations. First, patients with sideroblastic anemia were searched by questionnaire sent to hospitals with hematology department (493 hospitals) and pediatric hematology department (593 hospitals) asking for information about patients diagnosed as sideroblastic anemia (first investigation) over the past 10 years. Next, detailed clinical data of sideroblastic anemia patients were collected from the hospital based on responses to the first investigation (second investigation). Survey items were age of onset, gender, family history, hematological and biochemical findings, treatment, and cause of death. Then, genetic analysis of patients, who were diagnosed as CSA and MDS without chromosomal anomaly, was performed in cases for which genome sample was available (third investigation).

This study was approved by the ethics committee of Tohoku University Graduate School of Medicine, the center responsible for clinical and genetic analysis. Informed consent for the genetic analysis was obtained in all cases.

Diagnostic procedure

Ring sideroblasts were defined following the 2001 World Health Organization (WHO) classification. Sideroblastic anemia patients were diagnosed in the respective institutions. In all cases, bone marrow smears were investigated, and at least 15 % ring sideroblasts were confirmed by iron staining. Furthermore, diagnosis for RARS was made when dysplasia restricted to erythroid lineage in bone marrow was recognized. Diagnosis for RCMD was made when there is multilineage dysplasia. Thereafter, in the present study, RCMD correspond to refractory cytopenia with multilineage dysplasia and ringed sideroblasts (RCMD-RS) of the 2001 WHO classification. Diagnosis for CSA was made when the patient had a family history or the disease onset during infancy, or fulfilled the characteristic features of XLSA, such as onset at a young age, microcytic anemia, and responsiveness to Vit.B6.

Genetic analysis of patients with sideroblastic anemia

In the genetic analysis, mutations in *ALAS2*, *SLC25A38*, *GLRX5*, *ABCB7*, *PUS1*, and *SLC19A2*, which are known to be responsible for CSA, were examined in 14 cases of CSA and 10 cases of MDS. In addition, *SF3B1*, which was very recently reported to be mutated in sideroblastic anemia in MDS at a high incidence, were analyzed as well. Mutation analysis for the *ALAS2* gene was performed first in all candidates, and then the analysis proceeded to the other

genes if no mutations in *ALAS2* were detected. For mutation analysis of *ALAS2*, genomic DNA was extracted from the proband's peripheral blood using QIAamp DNA blood midi kit (QIAGEN, Valencia, CA, USA). The proximal promoter region [14], erythroid enhancer in intron 8 [15], and all exons and exon–intron boundaries of the *ALAS2* gene were amplified using ExTaq DNA polymerase (Takara Bio, Shiga, Japan) [16]. Amplified products were purified using a QIAquick gel extraction kit (QIAGEN) after agarose gel electrophoresis. They were then subjected to direct sequencing analysis using BigDye Terminator Cycle sequencing kit v1.1 with an ABI3100 genetic analyzer (Life Technologies Corp., Carlsbad, CA, USA). Mutation of the gene was confirmed by repeated polymerase chain reaction (PCR) followed by direct sequencing analysis. Genes other than *ALAS2* were sequenced by Hiseq2000® [6]. Briefly, genomic DNA was amplified using REPLI-g mini kit® (QIAGEN Science). After adjusting the concentration of amplified DNA, DNA from consecutive 12 samples was combined into one DNA pool, and the entire coding sequences were amplified by primers to which *Not*I linker was attached. The products were digested with *Not*I, and ligated with T4 ligase. Then, DNA was sonicated into ~200-bp fragments, and sequencing libraries were generated. Libraries were subjected to deep sequencing on Hiseq2000®. Sequencing data was analyzes as described previously. Detected mutations were validated by direct sequence.

Analysis of enzymatic activity of recombinant ALAS2 protein

For preparing recombinant ALAS2 proteins, complementary DNA (cDNA) encoding mature human ALAS2 protein was amplified using a following primer set (5′-GGTGGTCATATGATCCACCTTAAGGCAACAAAGG-3′and 5′-GGCATAGGTGGTGACATACTG-3′). The amplified cDNA was then treated with *Nde*I restriction enzyme and was cloned between *Nde*I and blunt-ended *Sap*I site of pTXB1 plasmid (New England Biolabs, Ipswich, MA, USA), resulting in pTXB-AEm. From this plasmid, mature ALAS2 protein was expressed as an inducible fusion protein with Intein and chitin-binding domain in *E. coli*. To obtain the mutant protein, the identified mutation was introduced into pTXB-AEm using PrimeStar Max site-directed mutagenesis kit (Takara Bio, Shiga, Japan). For expression and purification of wild-type and mutant ALAS2 proteins, *E. coli* BL21 (DE3) was transformed with each plasmid. The induction and purification of the recombinant proteins were performed using Impact system (New England Biolabs) according to manufacturer's instruction. Briefly, each recombinant protein was induced in *E. coli* with 0.1 mM IPTG at 25 °C for overnight. Then, cells were resuspended with lysis buffer (20 mM Tris–HCl pH8.5, 500 mM NaCl, 1 mM EDTA, 0.1 % Triton X-100, 1 mM PMSF, 1 μg/ml of antipain, pepstatin, and leupeptin). After the sonication and centrifugation, cleared cell lysates were incubated with chitin beads for 1 h at 4 °C, then washed with wash buffer (20 mM Tris–HCl pH8.5, 500 mM NaCl, 1 mM EDTA, and 0.1 % Triton X-100). Tag-free recombinant mature ALAS2 protein was obtained by on-column cleavage with 50 mM DTT in wash buffer at room temperature for 16 h. After the elution from the column, protein concentration was determined using Bio-Rad Protein assay reagent (Bio-Rad Laboratories, Inc., Hercules, CA, USA). The ALAS activity of each recombinant protein was measured in vitro, as described previously [8].

Statistical analysis

Results are presented as means±SD with the exception of the age of onset, which is expressed as the median. Statistical analysis was performed using Student's *t* test, and $P<0.05$ was taken to indicate statistical significance.

Results

Epidemiology of sideroblastic anemia

As of 31 January 2012, detailed data for 148 sideroblastic anemia, including MDS and secondary sideroblastic anemia, patients have been collected. Excluding 10 cases of refractory anemia with excess blasts (RAEB) and one case of sideroblastic anemia due to alcohol, the remaining 137 cases were classified as 18 cases of CSA, 47 cases of RARS, and 72 cases of RCMD. Of 18 CSA cases, 7 were already confirmed to be XLSA due to mutation of *ALAS2* before registration in this study, and the others were diagnosed as CSA based on family history or clinical findings, including responsiveness to Vit.B6 treatment. Clinical findings and family history, which suggest the porphyria, were not observed in any CSA patients.

Analysis of the pathology of congenital sideroblastic anemia

Laboratory data of CSA, RARS, and RCMD are shown in Tables 1 and 2. Median age at onset of CSA was younger than those of RARS and RCMD (19, 72.5, and 71 years old, respectively). Hemoglobin and mean corpuscular volume (MCV) values of CSA were significantly lower than those of RARS and RCMD cases (7.1 g/dl and 69.0 fl, 8.7 g/dl and 106.8 fl; and 8.3 g/dl and 106.5 fl, respectively). Serum iron level in CSA was significantly higher than that in RARS or RCMD (210.7, 162.8, and 171.1 μg/dl, respectively). These data have possibilities of reflecting the states of the iron over-loaded of CSA; however, as serum iron concentration is very instable and depends from different factors, this finding should be carefully evaluated.

Table 1 Clinical data of CSA, RARS, and RCMD (1)

	CSA (n=18)	RARS (n=47)	RCMD (n=72)	p-value (between CSA and RARS)	p-value (between CSA and RCMD)
Gender					
Male	17	33	44		
Female	1	14	28		
Median age at onset (year)	19.0 (±20.2)	72.5 (±10.4)	71.0 (±13.0)	<0.01	<0.01
White blood cells (/μl)	5547 (±2022)	4741 (±2561)	4105 (±1847)	0.24	<0.01
Red blood cells ($\times 10^4$/μl)	383.4 (±100.0)	245.6 (±45.6)	239.4 (±56.4)	<0.01	<0.01
Hemoglobin (g/dl)	7.1 (±2.21)	8.7 (±1.7)	8.3 (±1.8)	<0.01	0.02
Mean corpuscular volume (fl)	69.0 (±11.6)	106.8 (±9.0)	106.5 (±9.2)	<0.01	<0.01
Platelet ($\times 10^4$/μl)	28.5 (±12.62)	25.9 (±15.5)	23.9 (±24.1)	0.53	0.44
Reticulocyte (‰)	12.1 (±10.9)	17.7 (±10.8)	21.5 (±20.1)	0.07	0.05

When iron-related laboratory data were examined in transfusion independent cases (CSA, 13; RARS, 26; RCMD, 34), Serum iron level in CSA was tended to be higher than that in RARS or RCMD (210.6, 180.3, and 166.6 μg/dl, respectively), although the difference was not significant (p=0.07, data not shown). Serum ferritin level in CSA, RARS and RCMD were elevated in these transfusion independent cases (1,087.9, 898.1, and 732.2 ng/ml, respectively), suggesting that most of sideroblastic anemia patients were iron-overloaded before transfusion. There were no significant differences in other biochemical data among the three groups.

Chromosomal abnormalities of MDS

Data regarding cytogenetic abnormalities were available for all RARS patients and for 68 of 72 RCMD patients. Figure 1 shows the cytogenetic findings of RARS and RCMD. In RARS cases, chromosomal abnormalities were found in 17 patients (36.2 %). Abnormalities consisted of abnormality including +8 (3 cases), complex abnormality with deletion 5 (2 cases), and complex abnormality with 20q− (3 cases). Chromosomal abnormalities in RCMD were found in bone marrow samples from 27 RCMD patients (39.7 %). Abnormality including +8 was detected in nine cases (33.3 %) and abnormality of idic (X) (q13), associated with the *ABCB7* gene [17], was found in one case. In addition, −7, which was not identified in RARS, was identified in four RCMD patients (14.8 %).

Treatment and outcome

Analysis of the available data regarding treatment indicated that 17 of 47 RARS cases and 26 of 72 RCMD cases were administered Vit.B6 (data not shown). The effectiveness was judged according to the criteria of IWG [18], and one RARS patient obtained a major response, and three RARS patients and one RCMD patient obtained minor responses. Thus, 4 of 17 RARS patients and 1 of 26 RCMD patients responded to Vit.B6 treatment. However, improvement of Hb was not sustained in two RARS patients; Hb level gradually returned to or dropped below the pretreatment level. Therefore, Vit.B6 treatment may not be effective for MDS, or the effect if any may be very limited. The clinical outcomes of patients are shown in Supplemental Table 1. The median follow-up from the time of diagnosis in CSA patients was 30.5 months, and two patients died due to sepsis (one case) and cardiac failure (one case). One patient

Table 2 Clinical data of CSA, RARS, and RCMD (2)

	CSA (n=18)	RARS (n=47)	RCMD (n=72)	p-value (between CSA and RARS)	p-value (between CSA and RCMD)
Total bilirubin (mg/dl)	1.1 (±0.8)	1.3 (±0.9)	1.1 (±0.7)	0.47	0.78
AST (GOT) (IU/l)	33.0 (±24.3)	24.9 (±11.7)	27.9 (±20.8)	0.08	0.38
LDH (IU/l)	218.3 (±98.9)	263.5 (±119.2)	246.1 (±97.7)	0.16	0.28
CRP (mg/dl)	0.13 (±0.15)	0.40 (±1.16)	1.17 (±3.81)	0.37	0.30
Serum iron (mg/dl)	210.7 (±77.6)	162.8 (±73.6)	171.1 (±66.2)	0.03	0.04
UIBC (mg/dl)	80.4 (±113.6)	102.4 (±82.7)	79.9 (±60.7)	0.48	0.93
Ferritin (ng/ml)	1239.8 (±1306.8)	743.4 (±815.3)	804.3 (±990.2)	0.08	0.13

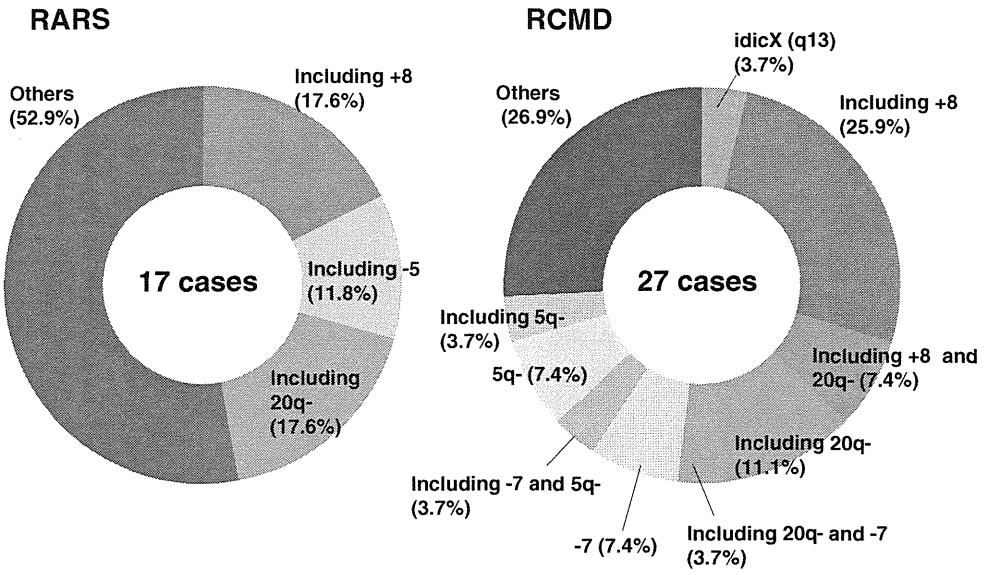

Fig. 1 Chromosomal abnormalities in RARS and RCMD. Data of chromosomal analysis in RARS and RCMD are shown. +8 was most common both in RARS and RCMD. -7 was only seen in RCMD

who died due to cardiac failure was heavily iron overloaded as defined by serum ferritin level, suggesting that cardiac complications may be caused by hemochromatosis. The median follow-up from the time of diagnosis in RARS patients was 23 months, and 6 patients (12.8 %) died due to pneumonia (two cases), evolution to leukemia (one case), and others (three cases). The median follow-up from the time of diagnosis in RCMD patients was 19.5 months, and 20 patients (27.8 %) died due to pneumonia (7 cases), cardiac failure (3 cases), evolution to leukemia (2 cases), sepsis (1 case), and others (7 cases). These results suggest that the prognosis of RCMD is worse than that of RARS.

Gene analysis of congenital sideroblastic anemia

Eighteen CSA patients were candidates for gene analysis; however, mutation analysis for genes responsible for CSA was not performed in four patients. One patient was diagnosed as having PMPS based on clinical findings, and DNA samples were not available for the remaining three patients. Therefore, gene analysis was performed in 14 of 18 CSA patients. Ten of these 14 patients were diagnosed as XLSA due to *ALAS2* mutation. Table 3 summarizes the results of gene analysis in XLSA. Case 2 (R411C), case 4 (D190V), case 6 (M567I), and case 7 (V562A) were reported previously [19–21]. Since amino acid substitution at Arg170, 411, and 452 were observed in plural patients, there are hot spots of mutation of *ALAS2* gene.

Patient with D190V (case 4), R170L (Case 10) and two patients with R452C (cases 3 and 5) did not respond to Vit.B6 treatment, whereas six patients responded to Vit.B6 treatment, although the increment of hemoglobin varied from 1.7 to 8.1 g. Interestingly, case 8 responded to Vit.B6 treatment, whereas case 10 did not, although both of them harbor the same mutation, R170L. Therefore, the activity of R170L mutant proteins was examined to determine the property, especially the Vit.B6 responsiveness. The enzymatic activities of wild type and R170L mutant protein were 7,193±138 nmol ALA/mg protein/h and 2,240±145 nmol ALA/mg protein/h, respectively, in the absence of PLP (Fig. 2). With an excess amount of PLP (100 μM) in the assay mixture, higher enzymatic activities were obtained with wild-type and mutant proteins (12,662±311 nmol ALA/mg protein/h and 7,700± 49 nmol ALA/mg protein/h, respectively) (Fig. 2). In addition, the enzymatic activity of R170C, which is another substitution at Arg170 found in this study, was also examined. As shown in Fig. 2, The enzymatic activity of mutant protein was significantly lower than wild-type protein without PLP (4,612± 87 nmol ALA/mg protein/h vs 7,193±138 nmol ALA/mg protein/h), and the activity was restored by addition of excess amount of PLP (100 μM) in the assay mixture. These in vitro data suggest that amino acid substitution at Arg 170, at least R170L and R170C, results in the decrease in enzymatic activity, but the decrease can be recovered by excess amount of PLP. The enzymatic activity of mutant proteins, which were identified in this study, is summarized in Table 3. The enzymatic activities of R411C, D190V, M567I, and V562A were referred from previous reports [19–21]. The levels of activity and PLP responsiveness in vitro were not correlated with clinical responsiveness to PLP in some cases. It is possible that the variety of mechanisms, such as the decrease in enzymatic activity of mutant ALAS2 protein, the changes of amount of *ALAS2* transcript, and physiological and environmental status of the patients, are responsible for the development of the disease.

Data for CSA patients other than XLSA are summarized in Table 4. Case 15 was diagnosed as PMPS. Gene analysis was not performed for cases 16 and 17; however, XLSA was strongly suspected because these patients were male and had microcytic anemia that was responsive to Vit.B6 treatment.

Table 3 Congenital sideroblastic anemia (XLSA)

Case number	Age at diagnosis (y.o.)	Gender	Position of *ALAS2* mutation	*SF3B1* mutation	Hb at onset (g/dl)	MCV at onset (fl)	Increment of Hb by Vit.B6 treatment (g/dl)	In vitro enzymatic activity of mutant protein[a]	
								Without PLP	With PLP
1	0	M	R170C	N/D	4.8	52.5	1.7	64.1 %	72.5 %[b]
2	20	M	R411C	N/D	4.8	52.5	5.2	11.9 %	25.1 % [19]
3	68	M	R452C	–	6.0	67.3	No effect	99.9 %	94.0 % [21]
4	17	M	D190V	N/D	8.9	66.9	No effect	98.6 %	98.5 % [20]
5	36	M	R452C	–	7.4	70.0	No effect	99.9 %	94.0 % [21]
6	36	M	M567I	N/D	6.5	64.4	3.4	38.1 %	25.2 % [21]
7	14	M	V562A	–	8.1	61.2	4.7	150.6 %	116.9 % [21]
8	31	M	R170L	–	4.1	50.8	8.1	31.1 %	60.8 %[b]
9	3	M	R411C	–	5.4	54.4	2.9	11.9 %	25.1 % [19]
10	62	M	R170L	N/D	8.0	73.9	No effect	31.1 %	60.8 %[b]

[a] % of WT

[b] Present study

ALAS2 mutations were not identified in cases 11, 12, 13, and 14. Therefore, mutations of *SLC25A38*, *GLRX5*, *ABCB7*, *PUS1*, *SLC19A2*, and *SF3B1* were examined in these cases; however, no mutations were identified in these cases. In contrast to other cases, case 18 was female and showed normocytic anemia. She was diagnosed with CSA due to family history; however, gene mutation analysis was not performed because DNA samples were not available. *SF3B1* gene mutation was examined in nine cases including five XLSA, however, no mutation was identified (Tables 3 and 4). On the other hand, *SF3B1* gene mutation was frequently detected in MDS-RS (Table 5).

Discussion

Because of its rarity, there have been few clinical and pathological investigations focusing on sideroblastic anemia. This study was performed to investigate the epidemiological and pathological characteristics of sideroblastic anemia. Based on the data of 137 patients, it was revealed that hemoglobin level in CSA was significantly lower than those seen in MDS, and serum iron level was higher in CSA compared to MDS. These results revealed that anemia in CSA is more severe than that in MDS at onset, although significant cases improved by Vit.B6 treatment. Reflecting the high incidence of XLSA in CSA, MCV level was significantly lower in CSA than MDS. These findings suggest that CSA should be strongly suspected rather than MDS, at least in Japan, in male patients exhibiting microcytic anemia and an elevated serum iron level.

MDS-RCMD is the most common form of acquired sideroblastic anemia. Chromosomal abnormalities were observed in 39.7 % of RCMD cases and 36.2 % of RARS cases. The types of chromosomal abnormality frequently observed in RCMD and RARS did not differ from those reported previously, such as +8, −7, 20q− and −5. Among them, +8 was observed in nine cases of RCMD (33.3 %). As the frequency of +8 in MDS was reported to be 6.5–16.7 %,

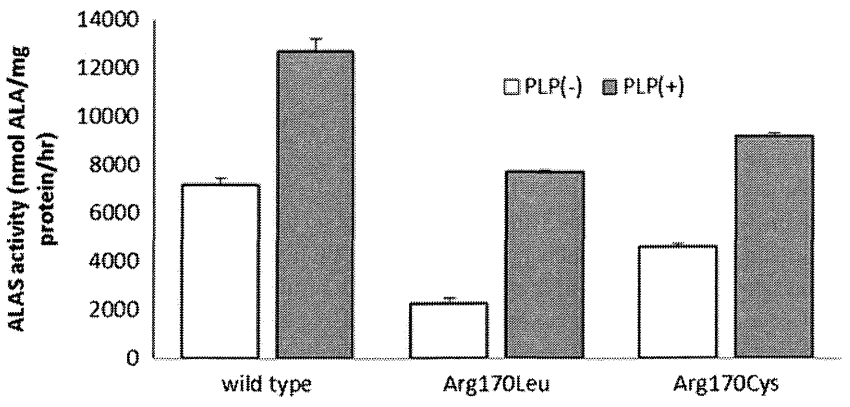

Fig. 2 Enzymatic activity of mutant ALAS2 proteins. Enzymatic activity of wild-type and mutant ALAS2 proteins was measured as described in Materials and Methods. Both of R170L and R170C ALAS2 mutant proteins showed decreased enzymatic activity; however, the activity was partially restored by the addition of PLP

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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