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

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

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

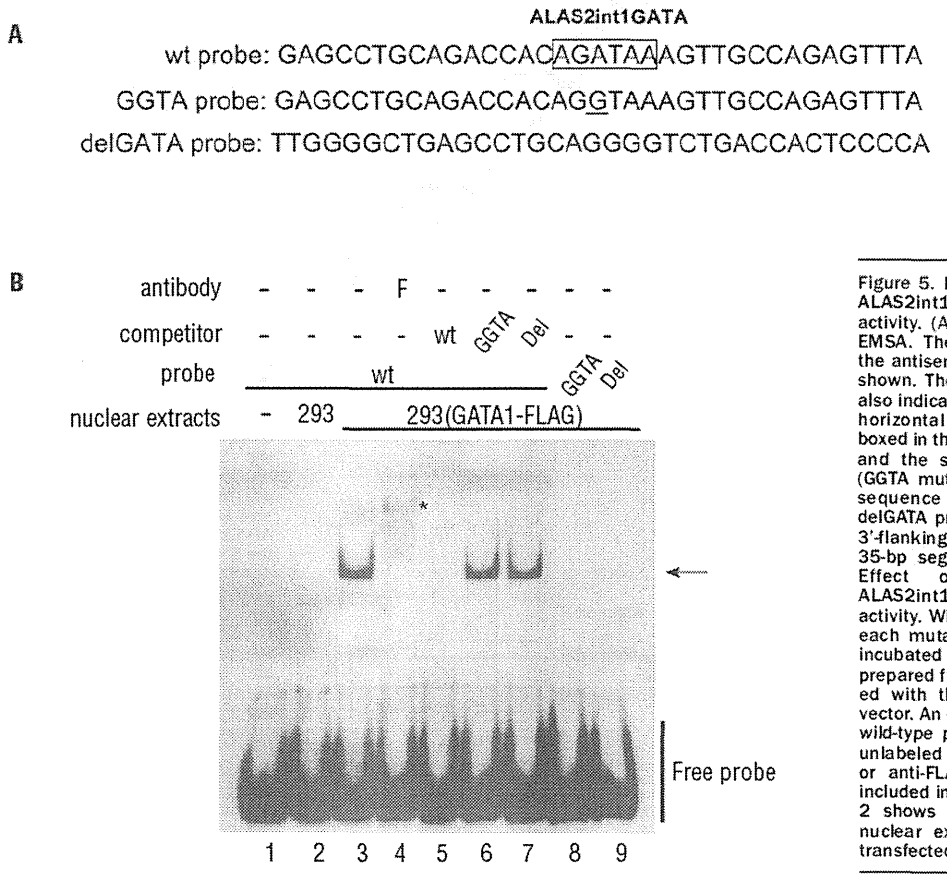
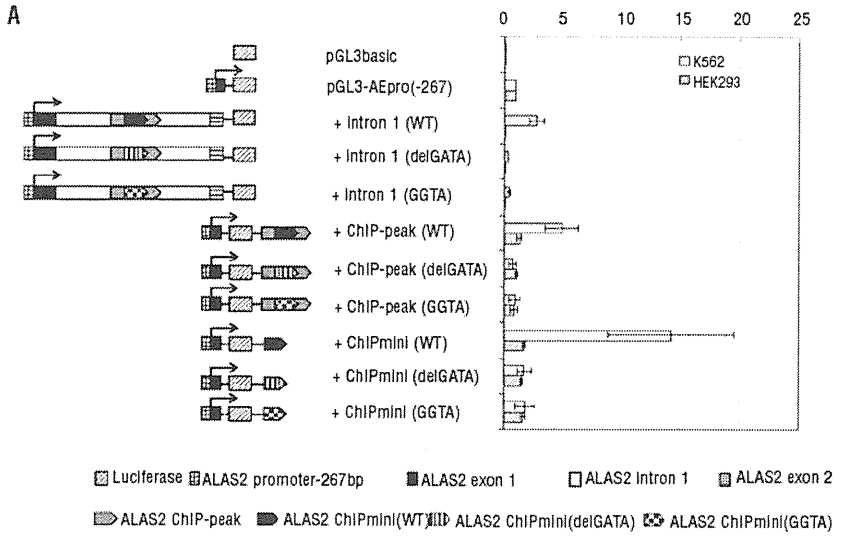


Figure 5. Effects of the mutations of ALAS2int1GATA on GATA1-binding activity. (A) DNA probes used in the EMSA. The nucleotide sequences in the antisense strand of the probes are shown. The position of each probe is also indicated in Figure 1B as the solid horizontal bar. ALAS2int1GATA is boxed in the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



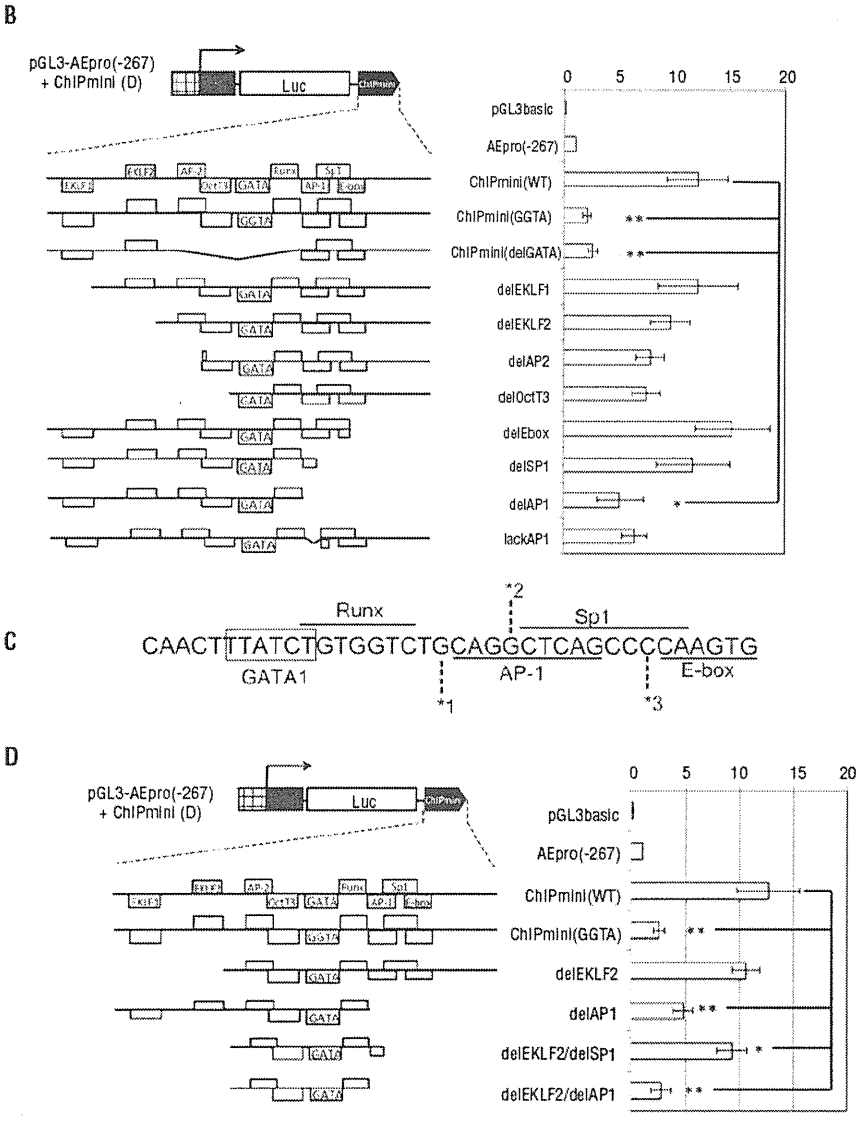


Figure 6. Identification of *cis*-elements essential for the erythroid-specific enhancer activity of ChIPmini. (A) Effect of each mutation of ALAS2int1GATA on the enhancer activity of *ALAS2* ChIPmini. The region corresponding to +intron1, ChIP-peak or ChIPmini, derived from proband 1 or proband 3, was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

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

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

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

Discussion

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

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

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

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

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Review Article

Pathophysiology and genetic mutations in congenital sideroblastic anemia

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

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

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

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

Genetic features and pathophysiology of CSA

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

Defects of heme biosynthesis

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

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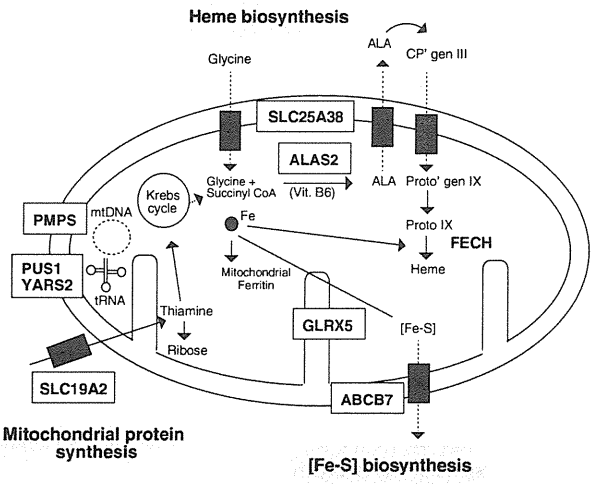

Fig. 1 Genes mutated in various types of congenital sideroblastic anemia. The pathogenic genes can be classified as those involved in (i) heme biosynthesis; (ii) iron–sulfur [Fe-S] cluster biosynthesis; and (iii) mitochondrial protein synthesis. ABCB7, adenosine triphosphate (ATP) binding cassette B7;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

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

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

Defects of [Fe-S] *cluster biosynthesis*

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

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

Table 1 Genetic features of congenital sideroblastic anemia

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

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

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

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

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

Abnormal mitochondrial protein synthesis

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

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

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

Epidemiological characteristics of causative genes for CSA

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

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

Conclusion

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

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Brief Report

RED CELLS, IRON, AND ERYTHROPOIESIS

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

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Key Points

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

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

Introduction

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

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

Study design

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

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

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

–, no case was found.

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

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

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

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

Results and discussion

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

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

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

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

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

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

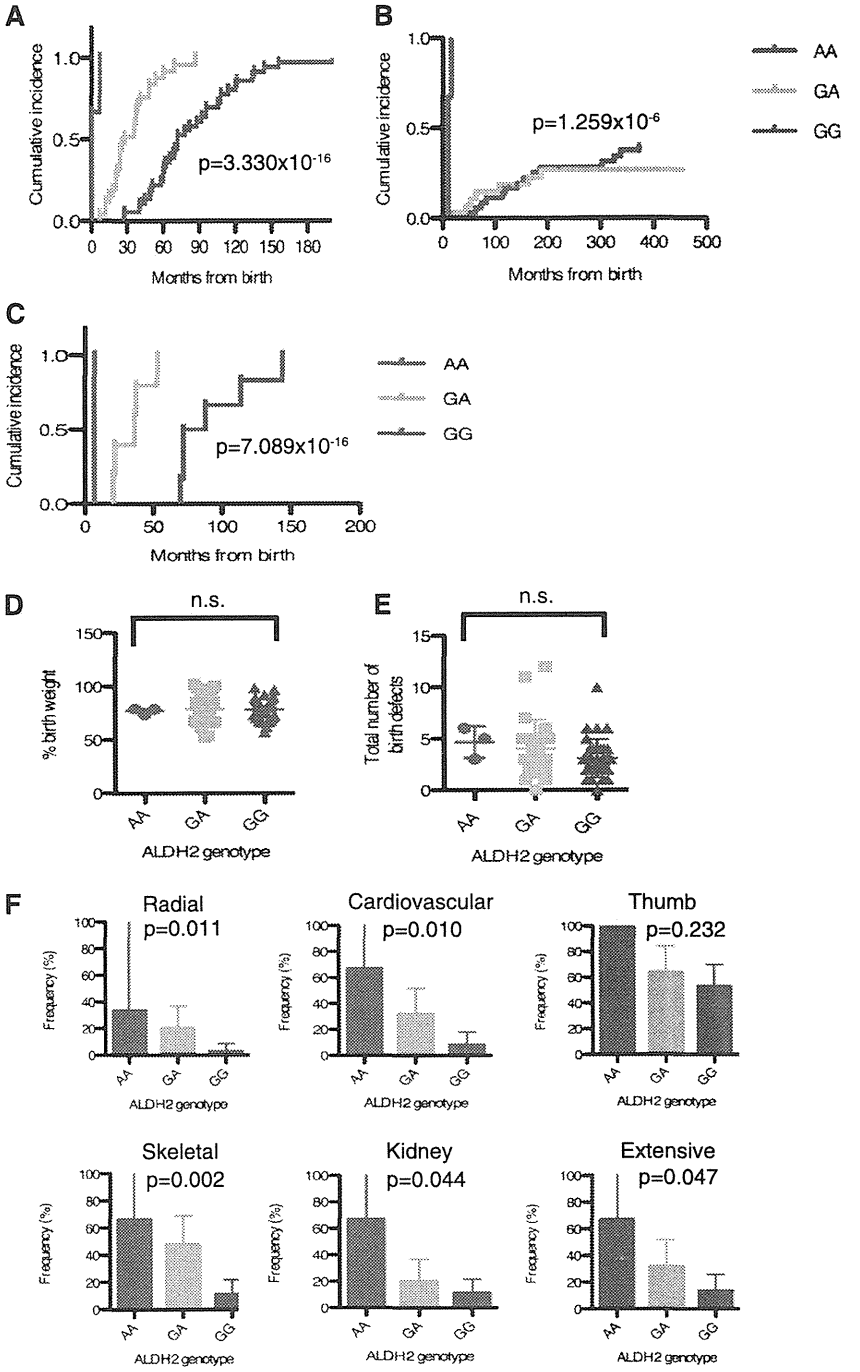

Figure 1. Effects of the ALDH2 deficiency on Japanese FA patients. (A-B) Cumulative incidence of BMF (A) or MDS/AML (B) were analyzed in 64 FA subjects. Numbers of AA, GA, and GG patients were 3, 25, and 36, respectively. (C) Cumulative incidence of BMF was analyzed in patients with confirmed biallelic *FANCA* mutations having protein truncations and/or large deletions (n = 12). Numbers of AA, GA, and GG patients were 1, 5, and 6, respectively. *P* values shown were calculated by the Gray test. In panel A, *P* values between genotypes were 8.625×10^{-7} (GG vs GA), 2.107×10^{-10} (GG vs AA), 1.259×10^{-6} (GA vs AA), respectively. In (B), the difference between GG and GA subjects was not significant ($P = .4564479$), whereas other statistical comparisons were highly significant (GG vs AA, 2.911×10^{-10}; GA vs AA, 8.813×10^{-8}). In panel C, the *P* values between GG and GA, GG and AA, or GA and AA were calculated as 0.001228433, 0.01430588, 0.02534732, respectively. (D) Percentage of birth weight or (E) total number of physical abnormalities (shown in supplemental Table 1) in 64 FA patients with 3 *ALDH2* genotypes. Birth weight was normalized to mean weight at gestational age in Japan. Mean and SEM are indicated. Birth weight records were missing for 3 patients (supplemental Table 1). There was no significant difference between the *ALDH2* genotypes (Kruskal-Wallis test). (F) Frequency (percentage) of cardiovascular, radial, thumb, skeletal, kidney, and extensive malformations in each *ALDH2* genotype. *P* values were calculated by the Cochran-Armitage test for trend, which detects statistical significance of effects across the genotypes. The error bars represent 95% confidence intervals.

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Authorship

Contribution: M.Y. and H.Y. examined DEB-induced chromosome aberrations, carried out MLPA testing, and analyzed clinical records; K.Y., Y.O., Y.S., K.C., H.T., S.M., S.K., and S.O.

performed WES and analyzed sequence data; A.H. validated exome data and carried out genotyping; A.H., M.Y., H.Y., K.M., J.N., and M.T. analyzed data; and M.Y., M.T., and K.M. wrote the paper.

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【第54回日本小児血液・がん学会学術集会】イブニング・セッション3：小児血液・がん医療における遺伝医療

遺伝性骨髄不全症候群における遺伝子解析と倫理的諸問題

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要　旨

遺伝性骨髄不全症候群（IBMFS）は，造血細胞の分化や増殖が先天的に障害され，血球減少を来す疾患群で，血球減少だけではなく種々の身体異常を伴う．従来は特徴的な身体所見や血液学的所見から臨床診断がされてきたが，ゲノム学の発展により，頻度の高い疾患については責任遺伝子が同定されてきている．IBMFSの中には，病初期には単一系統の血球減少であったものが2あるいは3系統の血球減少へと移行する疾患もあり，また骨髄異形成症候群や急性骨髄性白血病への進展や固形がんを発症する症例も稀ではない．数々の遺伝学的検査からなされる診断により，その疾患の治療法や予防法の適切な選択が可能となり，こうした遺伝学的検査は医師にとっても，患者にとっても重要な医療情報となってきている．一方，個人の遺伝情報の扱いには充分な配慮と対応が求められ，未成年者を対象とする小児科領域疾患では遺伝学的検査の実施時に考慮すべき点も多く，倫理的課題への取り組みの重要性が認識されるようになってきた．

キーワード：遺伝性骨髄不全症候群，遺伝子解析，Fanconi貧血，造血細胞移植，発がんリスク

Key words: inherited bone marrow failure syndrome, genetic analysis, Fanconi anemia, hematopoietic stem cell transplantation, cancer risk

I　はじめに

遺伝性骨髄不全症候群（IBMFS）[1]の中でも汎血球減少症をきたすIBMFSにはFanconi貧血（FA），Dyskeratosis congenita（DC），Shwachman-Diamond症候群などが含まれ，単一系統の血球減少は，赤血球系ではDiamond-Blackfan貧血（DBA），遺伝性鉄芽球性貧血，congenital dyserythropoietic anemia，好中球系では先天性重症好中球減少症，血小板系ではThrombocytopenia absent radiiなどがある．これらの疾患の特徴と遺伝形式を表1に示す．FAでは染色体断裂試験が，また，DCではテロメア長の測定がスクリーニング検査としてほぼ確立されており，DBAでは赤血球adenosine deaminase活性の測定が有用と考えられるが，その他のIBMFSには簡便なスクリーニング方法がなく確定診断は遺伝子解析にゆだねられることが多い．

このように遺伝学的検査はIBMFSの診療にとって重要な医療行為になっており，また患者家族にも大きな影響を与える個人情報を扱うために，十分な配慮と対応が求められる．2011年2月に日本医学会から「医療における遺伝学的検査・診断に関するガイドライン」が提唱された[2]．IBMFSはそのほとんどが稀少疾患であり，研究として行われる「研究検査」の形で行われており，これらは研究に関する指針に則って実施する必要がある．本稿ではIBMFSの中でも頻度の高いFanconi貧血を中心に病態生理，遺伝形式，疾患説明，治療法や遺伝子カウンセリング，生体試料の取り扱い等紹介すると同時に，こうした諸問題を共有できる機会となれば幸いである．

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II　Fanconi貧血の病態と遺伝

日本小児血液学会の全国登録によれば，わが国の年間発生数は5～10人で，出生100万人あたり5人前後である[3]．*FANCB*を除いて常染色体劣性の遺伝形式をとることから，キャリア頻度は，200～300人に1人と推定される．遺伝的に多様な疾患であり，現在までに16の責任遺伝子が同定されている（*FANCA, FANCB, FANCC, FANCD1, FANCD2, FANCE, FANCF, FANCG, FANCI, FANCJ, FANCL, FANCM, FANCN, FANCO, FANCP, FANVQ*）（表2）．これらのFAタンパク群は共通のネットワークにおいて働き，どれか1つの遺伝子産物が先天的に欠損すると，FA経路の機能不全のためDNA修復異常を引き起こし，FAとして発症する．FANCD1とFANCNのFAは幼少児期に白血病や脳腫瘍などを発症し重症病型を示す．さらにFANCD1，FANCJ，FANCNはそれぞれ家族性乳がん遺伝子タンパク群のBRCA2，BRIP1，PALB2と同一であり，ヘテロ接合体ではFAを発症しないが，発がんリスクの増加がみられる[4]．

FAの臨床像は多様で種々の身体異常を伴うが，全く異常がみられない症例もある．低身長，色黒の肌やカフェオ

表1　遺伝性骨髄不全症候群

疾患	血液病態	白血病への移行	固形腫瘍	遺伝形式
Fanconi anemia	再生不良性貧血	急性骨髄性白血病	扁平上皮癌	AR/(XR)
Dyskeratosis congenita	再生不良性貧血	急性骨髄性白血病	扁平上皮癌	XR/AD/AR
Scwachman-Diamond syndrome	再生不良性貧血	急性骨髄性白血病	–	AR
Diamond-Blackfan anemia	貧血	急性骨髄性白血病	肉腫，リンパ腫	AD
Hereditary sideroblastic anemia	貧血	–	–	XR/AR
Congenital dyserythropoietc anemia	貧血	–	–	AD/AR
Severe congenital neutropenia	好中球減少症	急性骨髄性白血病	–	AR/AD/XR
Thrombocytopenia absent radii	血小板減少症	急性骨髄性白血病	–	不明

AR；常染色体劣性，AD；常染色体優性，XR；X連鎖劣性

表2　Fanconi貧血遺伝子

相補群	頻度（%）	FA遺伝子
FA-A	～60	*FANCA*
FA-B	0.3	*FANCB*
FA-C	～15	*FANCC*
FA-D1	4	*FANCD1/BRCA2*
FA-D2	3	*FANCD2*
FA-E	1	*FANCE*
FA-F	2	*FANCF*
FA-G	～10	*FANCG/XRCC9*
FA-I	まれ	*FANCI*
FA-J	1.6	*FANCJ/BRIP1*
FA-L	0.1	*FANCL/PHF9*
FA-M	まれ	*FANCM/Hef*
FA-N	まれ	*FANCN/PALB2*
FA-O	まれ	*FANCO/Rad51*
FA-P	まれ	*FANCP/SLX4*
FA-Q	新規	*FANCQ/XPF*

Taniguchi et al, 2006[7]より改変引用

レ斑のような皮膚の色素沈着，上肢の母指低形成，多指症，橈骨欠損などの外表異常だけでなく，片腎，消化管や先天性心疾患などの内臓合併症や糖尿病などの内分泌異常も多い．進行性の再生不良性貧血を呈し，MDSやAMLへと移行する頻度が高く，固形がんも高頻度にみられ，頭頸部扁平上皮がん，食道がん，膣扁平上皮がんを含む女性生殖器のがんや肝細胞がんの占める割合が多い[5]．

III　どのような症例にFAの遺伝学的検査を行うのか

FAは，幹細胞レベルでの障害に基づく造血障害であり，免疫抑制療法の効果は期待できない．FAの患者にとって，現時点では，造血幹細胞移植のみが唯一治癒が期待できる治療法であるが，DNA修復異常を伴っているために前処置を弱めた移植を必要とする．その表現型は多様で，臨床像のみで本疾患を確定診断するのは不可能である．従って，造血不全の治療方針を決定するに当たり，小児や青年期に発症した再生不良性貧血患者に対しては，全例に染色体断裂試験を行い，FAを除外する必要がある．生下時より種々の身体異常を伴う症例や若年者において，頭頸部や食道，婦人科領域での扁平上皮がんや肝がんの発生がみられた場合や，MDSあるいは白血病の治療経過中に薬剤や放射線に対する過度の毒性がみられた場合にも，本疾患を疑い染色体断裂試験を行う必要がある．FAの造血不全に対する根本治療が造血細胞移植のみであるため，同胞がドナーとなる可能性があり，遺伝性疾患であるFAを否定するために，同胞ドナーの染色体脆弱試験が必要となる．また，同胞もFAである可能性もあるため，両親を含めて疾患に対する充分な理解とサポートを要する[6]．

IV　FAの遺伝子解析について

次世代シークエンサーの普及と共に，個人の全ゲノムが解読される時代となり，FAにおいても今まで不明であった症例の変異遺伝子が検出できるようになった．また，次々に新規遺伝子も発見され，今までに16の責任遺伝子が同定されてきた．これらの過程は多施設共同のゲノム解読作業となるため，各施設での倫理委員会での審議と連携が必要である．解析を行うに当たり，倫理的課題としてあげられることは，①インフォームド・コンセントあるいはアセントのあり方，②プライバシーの保護に伴う匿名化，検体の取り扱いや送付，患者・家系の識別の問題，③患者・家族に結果をどのように返すか，④匿名化データの公開のあり方，⑤同意の撤回，⑥検査後の生体試料の取り扱いなどである．

①に関しては，説明文書を作成し，FAの遺伝形式と遺伝とは何かの説明をまず行っている．特に保因者である両親には，すべての人は常染色体劣性遺伝病の病的遺伝子を6～7個はもっており，すべての人がみな何らかの疾患の保因者であること（両親の罪悪感を軽減する）の理解が得られるように努めている．また，すべてのFA患者に原因

遺伝子がみつかるわけではなく（検査の限界），現時点では遺伝子の違いで治療方法が異なることはないこと，将来的な発がん頻度等の違いがみつかれば，診療に生かすことができる可能性があることを加えて説明を行う．未成年である患者にも両親と相談の上，わかりやすい小児用の説明書を作成し，了解を得る努力を行っている．

②の点については，解析は多くの大学の研究者と共同作業で行うことを説明し，プライバシーの保護のため試料は匿名化番号をつけること，検体採取に際しては患者の身体的負担を最小限にとどめ，遺伝子解析の費用負担はないことも説明する．

③は最も慎重に対応すべき項目であり，患者には「知らずにいる権利」もあり，原則は希望があれば結果を伝えることとしている．特にFAでは極めて稀ではあるが，家族性乳がん遺伝子タンパク群である遺伝子が含まれており，その場合両親はFAの保因者であるためそのヘテロ接合体を保有している．FA患者の多くは染色体脆弱性と臨床症状で診断が確定的できるが，病状進行が早い症例では状態を早急に理解した上で治療方針の決定も急がれることや，発がんリスクの問題も常に抱えており，必要に応じて専門家による遺伝子カウンセリングの支援を受けられるように配慮する．

④⑤⑥についてはあらかじめ，説明および同意書の中に，研究成果の公表と研究から生じる知的財産権の帰属，研究協力の任意性と撤回の自由，遺伝子解析研究終了後の公的バンクを含む試料の保存（iPS細胞・株化細胞の作成など）と利用について記載され，まとめて同意を得る努力をしている．

V　FAの遺伝子解析を行った私たちの経験から

遺伝子解析により，患者・家族に確実な診断，治療法の選択，予後判定，合併症の回避など，有用な情報を与えることが重要である．一方，遺伝の説明は切り出しにくいと思っている医療者は多い．実際には，遺伝子解析の説明同意を得るに当たって，患者・家族からはむしろポジティブな印象が多く見受けられた．多くの家族・患者は遺伝についての情報を希望しており，医療者から遺伝の説明を受けることで納得する（感謝する）ことが多い．診断がつかない，どういう病気か解らないと言われる方が不安を与えることが明らかになった．遺伝子検査はほぼ全員が希望し，試料の保存・利用も検査を受けた全員が同意した．また，解析結果の開示も多くの家族が希望していることも判明した．共同研究施設を提示することで，多くの診療・研究施設が診断や治療に関わっていることに，驚きと感謝や希望を抱いていることも感じられた．個人のゲノム解析はインフォームド・コンセントが必須であるが，遺伝子情報の原理や意義は専門的で理解が困難であるため，患者や家族が十分な理解の上で同意できているとは限らず，医療者である私たちは，いつでも何度でも質問に応じて，病気を患者・家族と共有する姿勢が大切と思われる．また医療は日進月歩であり，今を乗り越えれば次の世代にはまた新しい治療法が開発されることを患者・家族が期待できる研究体制を継続していくことも重要である．

VI　おわりに

FAを始めとしてIBMFSにお蹴る原因遺伝子の特定は次世代シークエンサーの発展と共に急速に進み，診断や病態解明に大きく寄与している．そのほとんどのIBMFSの遺伝子診断が可能になることも遠い日ではない．同時にIBMFSの出生前診断の問題も浮上してくると予測される．技術的な可能性のみで安易に出生前診断を行うべきではなく，その適応には慎重な判断と倫理面での十分な審議が必要と思われる．

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CASE REPORT

Congenital dyserythropoietic anemia type 1 with a novel mutation in the CDAN1 gene previously diagnosed as congenital hemolytic anemia

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Abstract The congenital dyserythropoietic anemias (CDAs) are a heterogeneous group of genetic disorders of red cell production. They are characterized by ineffective erythropoiesis and dyserythropoiesis. Here, we present the clinical description and mutation analysis of a Japanese female with CDA type 1. She has long been diagnosed with unclassified congenital hemolytic anemia from the neonatal period. However, bone marrow morphology and genetic testing of the CDAN1 gene at the age of 12 years confirmed the afore-mentioned diagnosis. Thus, we should be aware of the possibility of CDA if the etiology of congenital anemia or jaundice cannot be clearly elucidated.

Keywords Congenital dyserythropoietic anemia · CDAN1 gene · Congenital hemolytic anemia

Introduction

The congenital dyserythropoietic anemias (CDAs) comprise a group of very rare hereditary disorders characterized by ineffective erythropoiesis and distinct morphological abnormalities of the erythroblasts in the bone marrow [1]. Morphological analysis is the first step in the diagnosis of all types of CDA, followed by confirmatory tests [2]. The diagnosis of CDAs can be delayed due to their rarity and lack of information (especially in non-severe cases) [3–5].

On the basis of the dysplastic changes observed in bone marrow erythroblasts by light and electron microscopy, the mode of inheritance and the associated dysmorphism, CDAs have been divided into 3 major types: CDA types 1, 2, and 3. Responsible genes have been identified for CDA type 1 (*CDAN1*) [6] and CDA type 2 (*SEC23B*) [7], not for CDA type 3.

In this brief report, we describe a unique case of CDA type 1 previously diagnosed as unclassified congenital hemolytic anemia. Marked erythroid dysplasia and the detection of a novel mutation in the *CDAN1* gene aided in accurately diagnosing the condition.

Case report

A 12-year-old female was referred to our hospital for further evaluation of persistent anemia after gastroenteritis. She had no family history of hemolytic anemia, was born at 39 weeks' gestation, and weighed 2,085 g at birth. Her initial symptom was severe jaundice at birth. She received three exchange transfusions during infancy, followed by erythropoietin administration for subsequent anemia up to the age of 1 year. At the age of 8 years, she experienced exacerbations of anemia, jaundice, and splenomegaly following mild gastroenteritis. Evaluation of her laboratory results at that point revealed low hemoglobin levels (10.6 g/dl), elevated mean corpuscular volume (MCV 101.3 fl), elevated bilirubin levels (total bilirubin 3.1 mg/dl, direct bilirubin 0.9 mg/dl), and undetectable haptoglobin (<10 mg/dl). The clinical and hematological features were suggestive of congenital hemolytic anemia; however, further investigation

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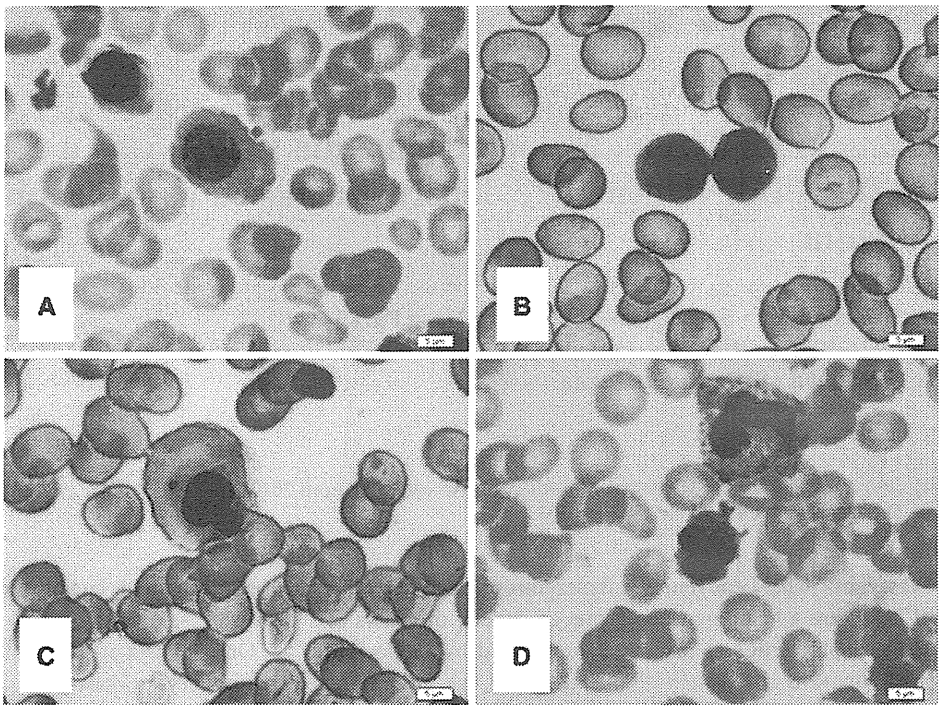

Fig. 1 Bone marrow morphology. **a** Megaloblastic changes, **b** nuclear bridging, **c** nuclear lobulations, and **d** multinuclearity (May–Giemsa staining, ×400)

[peripheral blood smear, osmotic fragility test, fraction of hemoglobin, isopropanol test, and red blood cell (RBC) enzyme activities] excluded the possibility of disorders of red cell membrane, thalassemias, unstable hemoglobinopathies, and red cell enzymopathies.

At the time of her first visit to our hospital, physical examination revealed mild splenomegaly and conjunctival pallor; she had no skeletal malformations (including distal limb anomalies). Laboratory evaluation revealed low hemoglobin levels (8.1 g/dl), normal MCV values (93.9 fl), normal bilirubin levels (total bilirubin 1.0 mg/dl, direct bilirubin 0.2 mg/dl), and mildly elevated serum ferritin levels (400.8 ng/ml). The levels of serum vitamin B12, folate, and iron were within the normal ranges. Furthermore, peripheral blood smear revealed anisocytosis and poikilocytosis (including teardrop-shaped poikilocytes), and schistocytes. Bone marrow examination revealed erythroid hyperplasia and marked erythroid dysplasia; megaloblastic changes, nuclear bridging, nuclear lobulations, multinuclearity were observed (Fig. 1). No significant features of dysplasia were observed in the myeloid or megakaryocytic lineages. To confirm the diagnosis, we conducted a mutational analysis that revealed a novel heterozygous frameshift mutation c.552_553 insG in exon 2, and another known [6] heterozygous missense mutation c.A1910G in exon 12 of *CDAN1* gene (Fig. 2); subsequently, we diagnosed her as a case of CDA type 1. One year after the diagnosis, her anemia resolved spontaneously (hemoglobin levels

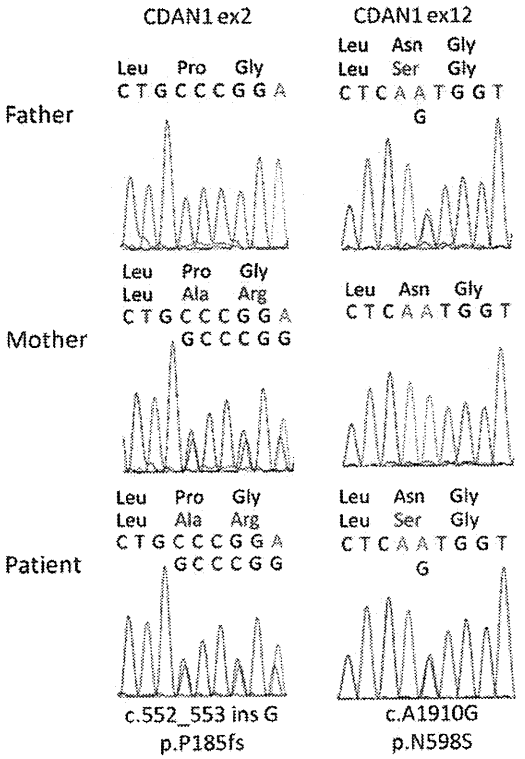

Fig. 2 The compound heterozygous mutation of the *CDAN1* gene

11.1 g/dl), but the ferritin levels remained relatively high (342.1 mg/ml); this required meticulous observation and follow-up.

Discussion

We report a 12-year-old female diagnosed with unclassified congenital hemolytic anemia with recurrent episodes of anemia and jaundice; subsequently, she was diagnosed with CDA type 1. CDA type 1 is inherited as an autosomal recessive disease. More than 150 patients have been described, mainly patients from Western Europe, the Middle East, India and Japan [8]. The anemia observed in CDA type 1 varies from mild to severe. About 50 % of neonates with CDA type 1 need at least one transfusion of erythrocytes, and some remain transfusion-dependent in the following years [9]. In most adolescents and adults, the need for transfusions is limited to aplastic crisis, pregnancy, periods of severe infections, or major surgery [10]. The anemia seen in CDA type 1 is usually macrocytic; in addition, peripheral blood smear showed other features of anisocytosis, poikilocytosis, and basophilic stippling [2]. Moreover, light microscopy of the bone marrow in CDA type 1 presents erythroid hyperplasia with abnormal precursors displaying a megaloblastoid appearance. Dysplastic signs include markedly irregular nuclei with frequent binucleate erythroblasts [11]. A particular diagnostic feature in CDA type 1 is thin, internuclear chromatin bridges between nearly completely separated erythroblasts.

Nevertheless, CDA should be diagnosed only after exclusion of other congenital anemias known to be associated with ineffective erythropoiesis and dyserythropoiesis [12]. Distinguishing CDA and the other congenital hemolytic anemias only on the basis of clinical course, laboratory data, and peripheral blood smear can be challenging. In CDA and the other congenital hemolytic anemia, symptoms of anemia and jaundice vary from mild to severe, with the most severe cases presenting in the neonatal period and milder cases presenting in adolescence or later stages in life. Abnormally shaped RBCs can appear in both the categories. Heimpel et al. [13] reported that in the German CDA Registry, the age of the 21 patients at the time of initial diagnosis of CDA type 1 ranged 0.1–45 years (median 17.3 years) and that 11 of 21 cases were previously misdiagnosed as congenital hemolytic anemia. Bone marrow examination might be often omitted, not usually performed, in pediatric cases with hemolytic anemia. In contrast, bone marrow examination is indispensible in case of CDAs because CDAs are diagnosed only after identifying distinct morphological abnormalities of the erythroblasts in the bone marrow.

Approximately, 90 % of patients with bone marrow evaluation suggesting CDA type 1 have mutations in *CDAN1* [6]. Most patients with a confirmed diagnosis of CDA type 1 demonstrate mutations of at least one allele from exons 6 to 28 within *CDAN1*; more than 30 unique mutations have been identified so far [6, 10, 13–17]. The majority of mutations in *CDAN1* are missense or nonsense, and only two frameshift mutations are known [10]. To our knowledge, c.552_553 insG in exon 2 is a novel frameshift mutation in *CDAN1*.

In summary, we report a Japanese female of CDA type 1. Bone marrow morphology and genetic testing in *CDAN1* gene was the key to accurate diagnosis. Taken together, when we encounter a patient whose clinical manifestations and laboratory results suggest the possibility of congenital hemolytic anemia but we cannot confirm the diagnosis, we should consider the possibility of CDA and bone marrow morphology and genetic testing should be conducted.

Conflict of interest The authors declare that they have no conflict of interest.

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