Ⅲ. 研究成果の刊行に関する一覧表

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雑誌 【欧文】

著者名	論文タイトル名	雑誌名	巻・号・ページ・出版年
Harigae H, Fruyama K	Hereditary sideroblastic anemia: pathophysiology and gene mutations.	Int J Hematol	92(3):425-431,2010
Tahara T, Yamamoto M Akagi R, Harigae H Taketani S	The low expression allele (IVS3-48C) of the ferrochelatase gene leads to low enzyme activity associated with erythropoietic protoporphyria.	Int J Hematol	92(5):769-771,2010
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Hereditary sideroblastic anemia: pathophysiology and gene mutations

Hideo Harigae · Kazumichi Furuyama

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Abstract Sideroblastic anemia is characterized by anemia with the emergence of ring sideroblasts in the bone marrow. Ring sideroblasts are erythroblasts characterized by iron accumulation in perinuclear mitochondria due to impaired iron utilization. There are two forms of sideroblastic anemia, i.e., inherited and acquired sideroblastic anemia. Inherited sideroblastic anemia is a rare and heterogeneous disease caused by mutations of genes involved in heme biosynthesis, iron–sulfur (Fe–S) cluster biogenesis, or Fe–S cluster transport, and mitochondrial metabolism. The most common inherited sideroblastic anemia is X-linked sideroblastic anemia (XLSA) caused by mutations of the erythroid-specific δ-aminolevulinate synthase gene (*ALAS2*), which is the first enzyme of heme biosynthesis in erythroid cells. Sideroblastic anemia due to *SLC25A38* gene mutations, which is a mitochondrial transporter, is the next most common inherited sideroblastic anemia. Other forms of inherited sideroblastic anemia are very rare, and accompanied by impaired function of organs other than hematopoietic tissue, such as the nervous system, muscle, or exocrine glands due to impaired mitochondrial metabolism. Moreover, there are still significant numbers of cases with genetically undefined inherited sideroblastic anemia. Molecular analysis of these cases will contribute not only to the development of effective treatment, but also to the understanding of mitochondrial iron metabolism.

Keywords Sideroblastic anemia · Iron · Mitochondria

1 Introduction

Red blood cells take up large amounts of iron during terminal differentiation for the production of hemoglobin. After import through the transferrin receptor, ferric iron (Fe^{3+}) is released from transferrin (Tf) in an acidified endosome, and is reduced to ferrous iron (Fe^{2+}) by ferrireductase. Ferrous iron is exported from the endosome to the cytosol via divalent metal transporter 1 (DMT1), and then transported to the mitochondria. The pathway from the endosomes to the mitochondria remains unclear, although it has recently been proposed that Fe–Tf-containing endosomes deliver iron directly to mitochondria, avoiding the toxicity of iron by bypassing the oxygen-rich cytosol. On reaching the mitochondria, iron is imported through an importer protein, mitoferrin 1 (Mfrn1; SLC25A38), which is a member of the solute carrier family localized in the inner mitochondrial membrane, and is used for heme synthesis and iron–sulfur (Fe–S) cluster biogenesis.

Heme is one of the two major products that require mitochondrial iron [1]. In mammals, eight enzymes are involved in the heme biosynthetic pathway. Heme synthesis starts in mitochondria with the condensation of glycine and succinyl CoA to form aminolevulinic acid (ALA), which is catalyzed by δ-aminolevulinate synthase (ALAS). ALAS consists of two isoforms, i.e., ALAS1, which is expressed ubiquitously, and ALAS2, which is

H. Harigae (✉)
Department of Hematology and Rheumatology,
Tohoku University Graduate School of Medicine,
1-1 Seiryo-machi, Aoba-ku, Sendai, Miyagi 980-8574, Japan
e-mail: harigae@med.tohoku.ac.jp

K. Furuyama
Department of Molecular Biology and Applied Physiology,
Tohoku University Graduate School of Medicine,
1-1 Seiryo-machi, Aoba-ku, Sendai, Miyagi 980-8574, Japan

expressed exclusively in erythroid cells. For hemoglobin synthesis in erythroid cells, the supply of protoporphyrin IX (PPIX) is regulated by ALAS2. ALA is then transported to the cytosol, and heme synthesis progresses successively in the cytosol following the second step. Coproporphyrinogen III (CoproIII), formed by uroporphyrinogen decarboxylase (UROD) at the fifth step, is imported into the mitochondria where the remaining three steps are performed. In the final step, reduced iron (Fe^{2+}) is inserted into PPIX by ferrochelatase (FECH), and then heme synthesis is completed.

Fe–S cluster is another major product that requires mitochondrial iron. As Fe–S cluster is an important electron transfer molecule, proteins containing Fe–S clusters are widely distributed and are involved in fundamental cellular activities, including multiple metabolic pathways, respiratory chain complexes, DNA synthesis, TCA cycle, and heme synthetic pathways.

When the utilization of mitochondrial iron is impaired by defects in heme synthesis, Fe–S cluster biogenesis, or disturbance of Fe–S cluster export, iron is accumulated in mitochondria. Excessively accumulated iron in mitochondria in erythroblasts becomes visible as iron granules by Prussian blue staining, and erythroblasts with perinuclear iron deposits are called ring sideroblasts. Such iron overload is likely to disturb cellular reduction–oxidation state and induce apoptosis, resulting in ineffective erythropoiesis. Anemia, which is characterized by this abnormal iron deposition in mitochondria in erythroblasts, is diagnosed as sideroblastic anemia. Reflecting the multiple mechanisms of disutilization of iron in mitochondria, the causes and clinical phenotypes of sideroblastic anemia are variable. This paper focuses mainly on the inherited form of sideroblastic anemia, and its pathophysiology is reviewed.

2 Inherited and acquired sideroblastic anemia

There are two forms of sideroblastic anemia, i.e., inherited and acquired sideroblastic anemia. Inherited sideroblastic anemia comprised heterogeneous phenotypes depending on the original function(s) of the mutated genes. To date, several genes responsible for inherited sideroblastic anemia have been identified. Responsible genes and phenotypes of each inherited sideroblastic anemia are summarized in Table 1. These genes play important roles in heme biosynthesis, Fe–S cluster biogenesis, or biology of mitochondria (Fig. 1). The incidence of inherited sideroblastic anemia is very rare, and the most frequent form is X-linked sideroblastic anemia (XLSA), which is caused by mutations in the erythroid-specific δ-aminolevulinate synthase gene (*ALAS2*). Bergmann et al. [2] examined the gene mutations of 83 probands of inherited sideroblastic anemia, and identified mutations of *ALAS2*, *SLC25A38*, mitochondrial DNA (mtDNA), and *PUS1*, in 37, 15, 2.5, and 2.5% of cases, respectively. Disease-causing mutations were not found in the remaining 43% of cases, suggesting that there are as yet unidentified genes, mutations of which cause inherited sideroblastic anemia.

Compared to inherited sideroblastic anemia, acquired sideroblastic anemia is relatively common. Acquired sideroblastic anemia is caused by drugs or alcohol; however, the best-known acquired sideroblastic anemia is refractory anemia with ring sideroblasts (RARS), a subtype of myelodysplastic syndrome (MDS). As MDS comprised heterogeneous groups, unique genetic abnormalities in RARS have not been identified. However, genes in which mutations cause inherited sideroblastic anemia may also be involved in the development of RARS. For example, a chromosome anomaly, isodicentric X chromosome with breakpoints in Xq13-idic(X)(q13), has been reported to be

Table 1 Inherited sideroblastic anemia

	Inheritance	Chromosome	Gene	Mutation	Treatment	Affected organ
XLSA[a]	X-linked	Xp11.21	ALAS2	Missense	Vit B6	–
XLSA/A[b]	X-linked	Xq13.1	ABC7	Missense	–	Nv
SA/GLRX5	Autosomal recessive?	14q32.13	GLRX5	Missense	?	L
SA/SCL25A38	Autosomal recessive?	3p22.1	SCL25A38	Missense	?	–
PMPS[c]	Maternal	Mitochondria	Mitochondria	Deletion	–	P, L, K, M, Nv
TRMA[d]	Autosomal recessive?	1q23.3	SCL19A2	Missense	Thiamine	P, H, Nv
MLASA[e]	Autosomal recessive?	12q24.33	PUS1	Missense	–	M

P pancreas, *L* liver, *K* kidney, *M* muscle, *Nv* nerve system

[a] X-linked sideroblastic anemia

[b] X-linked sideroblastic anemia with ataxia

[c] Pearson Marrow Pancreas Syndrome

[d] Thiamine-responsive megaloblastic anemia

[e] Mitochondrial myopathy and sideroblastic anemia

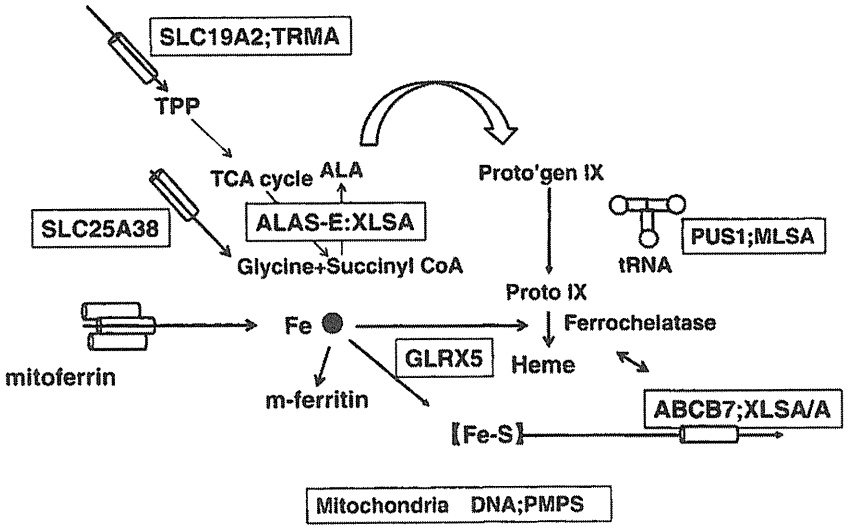

Fig. 1 Genes mutated in inherited sideroblastic anemia are involved in mitochondrial iron homeostasis. ALAS2 is the initial enzyme of heme biosynthesis in erythroid cells. The deficiency results in impaired heme biosynthesis. SCL25A38 is a transporter of glycine, a substrate for ALA synthesis. SCL19A3 is a transporter for thiamine, which is a cofactor of α-ketoglutarate dehydrogenase involved in the synthesis of succinyl CoA, a substrate for ALA synthesis. ABCB7 functions in Fe–S cluster transport, whereas GLRX5 functions in Fe–S cluster biogenesis. Deletion of mitochondrial DNA and failure of uridine modification may lead to impaired mitochondrial function

associated with RARS. By cytogenetic analysis, the *ABCB7* gene, which is responsible for X-linked sideroblastic anemia with ataxia (XLSA/A), was shown to be located at the distal region (74.1 Mb) on Xq13. As the breakpoint of idic(X)(q13) clusters in the region proximal to the position of the *ABCB7* gene, the *ABCB7* gene should be hemizygously deleted in idic(X)(q13). It is, therefore, possible that loss of the *ABCB7* gene induces sideroblast formation in RARS with idic(X)(q13) by the same mechanism as that in XLSA/A. In addition, it was recently reported that the level of *ABCB7* gene expression in CD34+ cells in RARS is significantly decreased compared to other MDS subtypes and healthy subjects, and the percentage of sideroblasts is correlated with the level of *ABCB7* gene expression [3]. Although the mutation of the *ABCB7* gene is not detected, cultured erythroblasts from RARS patients exhibited low levels of *ABCB7* gene expression. These findings suggest that aberrant *ABCB7* gene expression may be involved in the development of RARS. In contrast, the level of *ALAS2* gene expression, which is most frequently mutated in inherited sideroblastic anemia, was reported to be increased. Consistent with this, PPIX was also found to be elevated in RARS. Together with the lack of *ALAS2* gene mutations in RARS, these findings suggest that aberrant expression or mutation of genes encoding components of the heme synthesis pathway is unlikely to be responsible for RARS. Besides *ABCB7* gene, mtDNA mutations are suspected to be related to MDS. Since large deletion of mtDNA causes Pearson Marrow Pancreas Syndrome (Pearson syndrome), which is an inherited sideroblastic anemia, it is possible that mutations of mtDNA may play a role in the development of RARS. Indeed, Wulfert et al. [4] found variety of mutations of mtDNA, including tRNA, cytochrome oxidase, ATPase, and cytochrome *b*, in MDS patients. Since mutations specific to RARS were not identified, it is unclear whether these mutations are related to sideroblast formation. However, it is possible that dysfunction of these genes may cause impaired mitochondrial metabolism, leading to disutilization of iron in mitochondria.

3 Gene mutation and clinical features of inherited sideroblastic anemia

3.1 X-linked sideroblastic anemia

The erythroid-specific δ-aminolevulinate synthase, ALAS2 (or ALAS-E), which requires pyridoxal 5′-phosphate (PLP) as a cofactor to exhibit its activity, is the first enzyme in the heme biosynthetic pathway in erythroid cells. It was reported that ALAS activity is decreased in the bone marrow of inherited sideroblastic anemia cases, suggesting that impaired heme biosynthesis may induce the onset of certain of sideroblastic anemias. It was then revealed that *ALAS2* is located at Xp11.21, and a mutation of *ALAS2* was first reported in a male patient with sideroblastic anemia in 1992 [5], confirming that mutations of *ALAS2* are responsible for the development of XLSA. Subsequently, *ALAS2* mutations were confirmed in familial cases originally reported by

Rundles and Falls; therefore, "Rundles and Falls syndrome" is now recognized as XLSA. ALAS2 deficiency is thought to result in a decreased supply of PPIX, which causes disutilization of iron leading to excessive accumulation of iron in mitochondria in erythroblasts. The characteristics of XLSA are as follows: (a) inherited in an X-linked fashion; (b) hypochromic and microcytic anemia; and (c) systemic iron overload. The most important feature of XLSA is that more than half of all patients respond to oral pyridoxine treatment. The conformation of ALAS2 protein is thought to be altered by missense mutations, resulting in decreased affinity of PLP, so that administration of a large amount of pyridoxine restores the enzymatic activity, which in turn leads to improvement of anemia. As of 22 April 2010, at least 48 mutations in 74 pedigrees have been identified, including our unreported cases (Table 2). The sites of mutations are distributed from exon 5 through exon 11, encompassing exon 9, which contains the lysine residue responsible for PLP binding. The results of pyridoxine treatment were reported for 49 of these 74 pedigrees. Complete or marginal pyridoxine responsiveness was reported in 39 pedigrees, while pyridoxine refractoriness was reported in 10 pedigrees. In unresponsive cases, the *ALAS2* gene mutations create premature stop codons, or may change the stability of the enzyme, leading to reduction of ALAS2 protein expression level. In addition to mutations in the coding sequences, mutations in the promoter region of the *ALAS2* gene were also identified in pyridoxine-refractory XLSA patients, suggesting that the disease may occur due to impaired transcriptional regulation of ALAS2 gene. Of note, it should be highlighted that the emergence of anemia in XLSA does not always occur during childhood. We reported a case with development of sideroblastic anemia at the age of 81 years while undergoing hemodialysis [6]. The diagnosis of XLSA was made based on a mutation in the fifth exon of the *ALAS2* gene, which decreased enzymatic activity of recombinant mutated ALAS2 protein. The clinical course of improvement of anemia by pyridoxine treatment supports the diagnosis of XLSA. The very late onset in this case of XLSA suggests that environmental stress, including nutritional deficiencies or, as in this case, hemodialysis therapy, may induce the emergence of disease in occult XLSA cases.

The establishment of an animal model of XLSA has been attempted by modification of *ALAS2* gene. *Alas2*-null mice died on embryonic day 11.5 due to severe anemia. *Alas2*-null mice lacked hemoglobinized erythroblasts, and large amounts of iron were accumulated in these erythroblasts [7]. However, iron accumulation was observed in the cytoplasm and typical sideroblasts were not seen. Definitive erythroblasts generated from *Alas2*-null ES cells in vitro were also not hemoglobinized, and again iron was accumulated in the cytoplasm, but not mitochondria, although biological changes in erythroblasts, including apoptosis and oxidative status induced by excess of iron, were observed [8]. This phenotypic discrepancy between ALAS2-deficient erythroblasts in human disease and those in the mouse model may be due to the species-specific background differences; however, ring sideroblast formation was observed in the fetal liver of *Alas2*-null mice partially rescued by the human *ALAS2* transgene. These results suggest that the formation of ring sideroblasts

Table 2 Amino acid substitution of ALAS2 in patient with XLSA

Ex.	Substitution		No. of pedigree		Ex.	Substitution	No. of pedigree	Ex.	Substitution		No. of pedigree	
4	L107P		1		6	R227C	1	9	R448Q		3	
5	M154I		1			S251P	1		R452	C	6 (3)	16
	K156E		1		7	D263N	1			S	2	
	D159	N	1	2		C276W	1			H	8 (2)	
		Y	1			G291S	1		R458H		1	
	T161A		1			K299Q	1		I476N		1	
	F165L		2			V301A	1	10	Y506-fs		1	
	R170	S	1	7	8	D351R	1		T508S		1	
		C	2 (1)			T388S	1		R517	C	1	2
		L	3 (2)		9	C395Y	1			G	1	
		H	1			G398D	1		P520L		2	
	A172T		1			R411C	4 (1)		H524D		1	
	D190V		1			G416D	1	11	R559H		1	
	Y199H		1			M426V	1		R560H		3	
	R204	Q	1			R436W	1		V562A		1	
		stop	1						M567I		1	
									S568G		2 (1)	

As of 22 April 2010, at least 48 mutations in 74 pedigrees have been known. Our unreported cases were included, but promoter mutation was not included in this list

requires low levels of ALAS2, and complete loss of ALAS2 activity may shut off the iron transport into mitochondria. To clarify the mechanism of sideroblast formation and development of anemia in XLSA, the development of a viable mouse model is desirable.

3.2 Inherited sideroblastic anemia due to *GLRX5* mutation

Fe–S clusters function as active centers for electron transfer in enzymes involved in various cellular activities. Most proteins involved in Fe–S biogenesis are located in mitochondria in eukaryotes. Glutaredoxin 5 (GLRX5) is one of these proteins, and loss of grx5 function in yeast results in mitochondrial iron accumulation. In addition, GLRX5 is essential for Fe–S biogenesis in humans. The zebrafish mutant *shiraz* harboring GLRX5 deletion has severe anemia due to impaired heme synthesis caused by insufficient biogenesis of mitochondrial Fe–S clusters. The homozygous mutation of *GLRX5* was recently identified in an inherited sideroblastic anemia patient [9]. The mutation identified in the patient was shown to interfere with the splicing of intron-1, resulting in decreased *GLRX5* mRNA level. The patient presented with moderate anemia, hepatosplenomegaly, a reduced number of ring sideroblasts in the bone marrow, and iron overload.

Based on the analyses of zebrafish mutant *shiraz* and clinical data of the patient, the mechanism of sideroblast formation caused by *GLRX5* mutation was speculated as follows (Fig. 2). (1) Fe–S cluster biogenesis is decreased due to *GLRX5* mutation. (2) Decreased level of Fe–S clusters prevents the conversion of IRP1 (iron regulatory protein 1) to cytosolic aconitase, leading to increased level of IRP1 activity. (3) IRP1 represses translation of ALAS2 protein and stabilizes transferrin receptor mRNA through IRE. (4) Decreased level of ALAS2 results in deficiency of PPIX, whereas increased level of transferrin receptor results in stimulation of iron import. (5) Increased cellular iron level and disutilization of iron lead to iron deposition in mitochondria. (6) Deficiency of heme synthesis due to ALAS2 deficiency consequently stabilizes IRP2, resulting in repression of ALAS2 protein translation and stabilization of transferrin receptor mRNA. This successive loop through IRP for iron deposition suggests that the mechanism of sideroblast formation by GLRX5 deficiency is common with that of ALAS2-deficient activity.

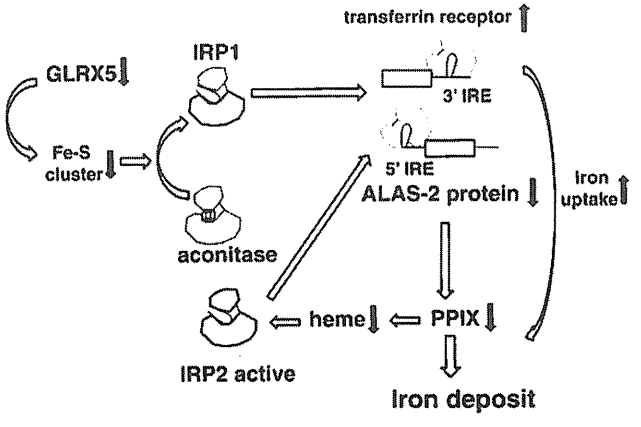

Fig. 2 Mechanism of sideroblast formation due to GLRX5 deficiency. The mechanism of sideroblast formation by mutation of GLRX5 is speculated to involve heme deficiency and increased iron import by IRP activation, sharing that by mutation of ALAS2

3.3 X-linked sideroblastic anemia with ataxia

X-linked sideroblastic anemia with ataxia is a rare sideroblastic anemia inherited in an X-linked fashion similar to XLSA. XLSA/A patients exhibit early onset cerebellar ataxia, typically in the first year of life. The ataxia is usually stable or slowly progressive. The ataxia observed in XLSA/A patients may be related to the mitochondrial damage generated by iron loading in neural cells. The anemia of XLSA/A is hypochromic and microcytic. Anemia is typically mild, and is not responsive to pyridoxine. In addition, systemic iron overload is not observed. From the results of molecular analysis, mutation of the *ABCB7* gene, which is located at Xq13.1, was found to be responsible for the development of XLSA/A. To date, three missense mutations (Ile400 Met, Glu 433Lys, and Val 411Leu) of ABCB7 have been reported [10].

ABCB7 is a transporter protein, containing an ATP binding cassette, located in the inner membrane of mitochondria. In yeast deficient for ATM1p, which is a homolog of *ABCB7*, iron accumulates in mitochondria, and human ABCB7 rescues this phenotype. As ABCB7 is thought to be involved in export of Fe–S complexes, it is possible that impaired activity of ABCB7 affects the function of various molecules containing Fe–S clusters. Although the consequences of deficiencies of both GLRX5 and ABCB7 protein could be the same with regard to decreased cytosolic Fe–S clusters, the mechanism of iron accumulation in mitochondria induced by ABCB7 deficiency appears to be different from that by GLRX5, because erythrocyte protoporphyrin levels in XLSA/A patients were reported to be normal/high, suggesting normal ALAS2 function. It was shown that the interaction of ABCB7 with FECH increases the activity of FECH, which is the final enzyme in the heme synthetic pathway, and so it is possible that deficiency of ABCB7 affects the activity of FECH; however, FECH activity was normal in murine models. Thus, although iron may primarily accumulate and PPIX exists in mitochondria, accumulated iron is not available to FECH for heme synthesis in patients with

XLSA/A. The precise mechanism of mitochondrial iron accumulation in these patients remains to be elucidated.

3.4 Inherited sideroblastic anemia due to *SLC25A38* mutation

Recently, Guernsey [11] carried out SNP-based genome-wide scans in subjects with familial or sporadic congenital sideroblastic anemia, and identified several mutations of the *SLC25A38* gene. The pattern of mutation varies, including nonsense mutation, frameshifts, splice acceptor site mutation, and missense mutation, and inheritance is autosomal recessive.

SLC25A38 is a member of the inner mitochondrial membrane transporters, highly expressed in erythroid cells. The phenotypic abnormality of the yeast with deletion of the *SLC25A38* ortholog was consistent with the defect of heme biosynthesis, and the phenotype was rescued by supplementation with either glycine or 5-aminolevulinic acid (ALA), a substrate and product of ALAS, respectively. Therefore, it is hypothesized that SLC25A38 facilitates ALA production by transport of glycine into mitochondria. From the findings both in this study and recent analysis by Bergmann [2], the incidence of inherited sideroblastic anemia due to *SLC25A38* mutation appears to be high in inherited sideroblastic anemia. Therefore, it is necessary to examine the mutation in Japanese patients with sideroblastic anemia whose *ALAS2* mutation has not been identified or in cases of RARS.

3.5 Pearson Marrow Pancreas Syndrome (Pearson syndrome)

Pearson syndrome is a rare sideroblastic anemia accompanied by metabolic acidosis, ataxia and exocrine pancreatic insufficiency [12]. The disease is usually fatal, and patients die during infancy. Anemia is normomacrocytic, and neutropenia and thrombocytopenia are sometimes present. Pearson syndrome is caused by the deletion or duplication of mitochondrial DNA. The disease is usually sporadic, and occurs de novo. The mechanism of sideroblast formation in Pearson syndrome remains unclear. Deletion of mitochondrial DNA may result in deficiency of respiratory chain complex, including respiratory complex I (NADH dehydrogenase), complex IV (cytochrome *c* oxidase), and complex V (ATP synthase). Iron should be in the reduced state, "ferrous (Fe^{2+}) iron," when incorporated into PPIX by FECH in the final step of heme synthesis. It is speculated that cytochrome *c* oxidase functions to keep iron in the reduced state, and therefore the defect of cytochrome *c* oxidase fails to supply ferrous (Fe^{2+}) iron for FECH. Thus, iron is not utilized for heme synthesis, which results in mitochondrial iron overload.

3.6 Thiamine-responsive megaloblastic anemia

Thiamine-responsive megaloblastic anemia (TRMA) represents sideroblastic anemia with systemic symptoms, including insulin-dependent diabetes mellitus and neural hearing loss. TRMA is a rare autosomal recessive disorder, usually diagnosed during childhood. The anemia is macrocytic accompanied with megaloblastic features. The disease is responsive to thiamine supplementation, but not to folate, Vit B12, or pyridoxine. The gene responsible for TRMA is *SLC19A2*, which encodes a thiamine transporter [13]. It is not clear how mutations of *SLC19A2* are involved in sideroblast formation. As thiamine is required as a cofactor for α-ketoglutarate dehydrogenase, which is involved in the synthesis of succinyl CoA, a substrate for ALA, mutations of *SLC19A2* may induce sideroblasts by deficiency of heme. However, this speculation has yet to be confirmed; because there was a report that erythrocyte porphyrin level was not decreased in TRMA patients.

3.7 Mitochondrial myopathy and sideroblastic anemia

Mitochondrial myopathy and sideroblastic anemia (MLASA) is an extremely rare autosomal recessive disease characterized by myopathy, lactic acidosis, and sideroblastic anemia [14]. The disease is caused by molecular defects of the pseudouridylate synthase 1 gene (*PUS1*), which functions in pseudouridine modification of tRNAs [15]. Pseudouridine is an isomer of the nucleoside uridine, and is formed by enzymes called pseudouridine synthases. Pseudouridine is known to affect the structure of tRNA and to strengthen base pairing; therefore, failure of pseudouridine modification may lead to aberrant translation. There are not only cytoplasmic tRNAs but also mitochondrial tRNAs, and these mitochondrial tRNAs contain potential substrates for PUS1. Therefore, the mutation of *PUS1* may result in impaired biology of mitochondria.

4 Conclusion

Inherited sideroblastic anemia is a rare hematological disorder; however, it is very important to identify the genes, mutations of which are responsible for the disease, as analyzing their function will improve our knowledge of mitochondrial iron metabolism. Furthermore, if the mechanism of iron accumulation in mitochondria is common between inherited and acquired sideroblastic anemia, findings obtained in the study of inherited sideroblastic anemia could be helpful to understand the pathophysiology of acquired sideroblastic anemia, including RARS, and contribute to the establishment of specific treatment strategies.

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LETTER TO THE EDITOR

The low expression allele (IVS3-48C) of the ferrochelatase gene leads to low enzyme activity associated with erythropoietic protoporphyria

Tsuyoshi Tahara · Masayoshi Yamamoto · Reiko Akagi · Hideo Harigae · Shigeru Taketani

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Erythropoietic protoporphyria (EPP) is an autosomal-dominant inherited disorder characterized biochemically by the excess accumulation and excretion of protoporphyrin, an intermediate precursor of heme biosynthesis. The enzyme abnormality that underlies protoporphyrin accumulation in EPP is a defect of ferrochelatase (FECH). Patients with EPP are clinically characterized by painful photosensitivity in skin and some (5–10%) exhibit liver failure due to massive hepatic accumulation of protoporphyrin [1, 2]. After we demonstrated the structure of the human *FECH* gene [3], more than 100 different kinds of molecular defects of FECH have been reported throughout the world. It has been reported that the low expression of a wild-type allelic variant *trans* to a mutated FECH allele is generally required for clinical expression of EPP [4]. According to this background, Gouya et al. [5] have found that the presence of a C at IVS3-48 in the human *FECH* gene causes the low expression of FECH. This intronic single nucleotide polymorphism (SNP) of the *FECH* gene, IVS3-48C/T transition, is key to the EPP phenotype. It is suggested that partially aberrant splicing of pre-mRNA by IVS3-48C is responsible for the clinical manifestations of EPP, although change in the enzyme activity has not been examined. Here, we report mutations of the *FECH* gene associated with IVS3-48C in five Japanese EPP patients. We found that the FECH activity of peripheral blood lymphocytes with IVS3-48C/C was <50% of that with IVS3-48T/T suggesting that the variations of the activity in patients with EPP could be based on the different levels of control.

T. Tahara · M. Yamamoto · S. Taketani (✉)
Department of Biotechnology,
Kyoto Institute of Technology,
Kyoto 606-8585, Japan
e-mail: taketani@kit.ac.jp

R. Akagi
Department of Pharmacy, Faculty of Pharmacy,
Yasuda Women's University, Hiroshima 731-0153, Japan

H. Harigae
Department of Hematology and Rheumatology,
Tohoku University Graduate School of Medicine,
Sendai, Miyagi 980-8574, Japan

1 Mutation of the *FECH* gene in patients with EPP

We have diagnosed five patients with EPP in Japanese hospitals (Table 1). All patients suffered photosensitivity and three of them (patients 3, 4 and 5) developed hepatic dysfunction and died. Biochemical analysis of all patients showed marked elevation of protoporphyrin in erythrocytes. The FECH activity in peripheral blood lymphocytes of EPP patients decreased to 19–39% that of the control. After informed consent for all examinations had been obtained from patients and their families, blood samples were collected for genetic analysis. The total RNA was isolated by the guanidine thiocyanate method from lymphocytes or Epstein–Barr virus-transformed lymphoblastoid cells. cDNAs were synthesized with oligo(dT) primer using ReveTra Ace (Toyobo Co. Ltd., Tokyo, Japan). The entire FECH protein-coding region was amplified by PCR using two synthetic primers, 5′-GAGGCTGCCCAGGC A-3′ and 5′-TTTGCCTAACGCCACGGGGT-3′. The DNA fragments were ligated into pGEM-T vector (Promega Co., Madison, WI). Several plasmids-carrying FECH cDNA from a patient were isolated and the inserted DNAs were analyzed by sequencing. We found mutations in cDNAs. To confirm the mutation, we tried to analyze mutations of the *FECH* gene; namely, genomic DNA was isolated from whole blood cells. Regions containing molecular defects

Table 1 Characterization of Japanese patients with EPP in terms of phenotype and genotype

Patient no.	Sex	Age	Symptoms	Protoporphyrin in blood (μg/dl RBC)	Mutation in FECH	Genotype of normal allele IVS3-48
1	M	23	Photosensitivity	1,424	IVS4(−4)a>g	C
2	M	33	Photosensitivity	9,274	Δ5b (751–755)	C
3	M	41	Photosensitivity liver failure	12,574	T557C (I186T)	C
4	M	27	Photosensitivity liver failure	8,779	Δ16b (574–589)	C
5	M	36	Photosensitivity liver failure	9,127	IVS9(+1)g>a	C

found in FECH cDNA were amplified with primers as previously reported [6]. The amplified DNAs were directly sequenced. Then, we identified five different mutations that were the same as those previously reported for Japanese and European patients [2]. The common mutations between Asians and Caucasians can be ascribed to their common ancestry.

2 Relation of IVS3-48T/C of the *FECH* gene to Japanese EPP

The IVS3-48C/T transition of the *FECH* gene from EPP patients and their families was also analyzed. To amplify the DNA of the intron 3–exon 4 boundary (278 bp), the primers 5′-TCTACAACAAGAGAGCTGGC-3′ and 5′-ATCCTG CGGTACTGCTCTTG-3′ were used. Five Japanese EPP patients presented in this study were found to exhibit IVS3-48C of the normal allele (Table 1), which is consistent with the previous studies of Japanese [7], Caucasian and Asian EPP patients [2]. On the other hand, all carriers ($n = 4$) in their families were found with IVS3-48T of the normal allele. Other possible low expression alleles of the *FECH* gene, such as −251 G/A and IVS1-23C/T transitions linked to the disease [4], were also examined for the five EPP families, but the examination was not conclusive. Thus, the variation of IVS3-48C/T transition in the *FECH* gene may explain the difference in the residual enzyme activities in asymptomatic and symptomatic mutant carriers. Alternatively, because EPP development requires with the mutated allele of the *FECH* gene as well as the allele with IVS3-48C, it can be said that EPP is a recessive-inherited disease in a broad sense. We examined the relationship of decreased FECH activity with the genotype of the *FECH* gene, including IVS3-48C/T transition. After the isolation of peripheral blood lymphocytes of EPP patients and Japanese healthy controls, we examined the FECH activity by the formation of zinc-mesoporphyrin [8]; namely, homogenates from lymphocytes were incubated with mesoporphyrin (10 nmol), zinc acetate (40 nmol), Tween 20 (0.01%), and sodium palmitate (400 μg/mL) in 100 mM Tris–HCl, pH 8.0. The formation of Zn-mesoporphyrin was determined by

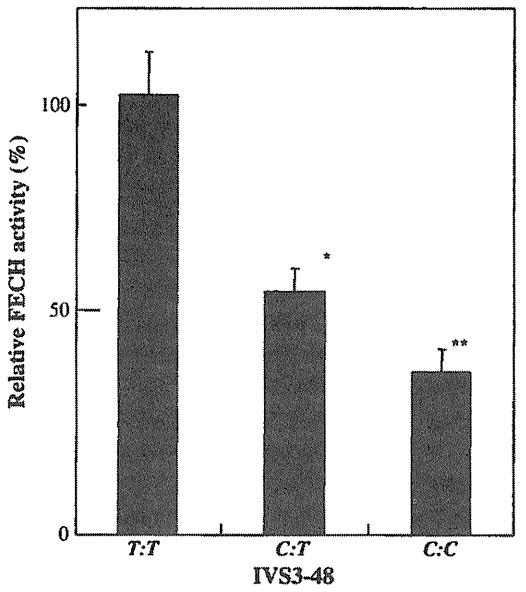

Fig. 1 The FECH activity in peripheral blood T lymphocytes from healthy controls. Lymphocytes were isolated from healthy volunteers with IVS3-48T/T (*T:T*) ($n = 9$), IVS3-48C/T (*C:T*) ($n = 10$) and IVS3-48C/C (*C:C*) ($n = 4$) of the *FECH* gene. The FECH activity was measured using homogenates. The activity of 100% is equivalent to 67.2 ± 6.5 nmol Zn-mesoporphyrin formed/10^6 cells/h at 37°C with IVS3-48T/T. $^*P < 0.01$, *C:T* versus *T:T*; $^{**}P < 0.005$, *C:C* versus *T:T*

HPLC with 5C18-5AR column (4.6 × 150 mm) (Nacalai Tesque, Kyoto, Japan). As shown in Fig. 1, the highest activity was observed in the genotype with IVS3-48T/T, a moderate level was shown with IVS3-48C/T, and the lowest level was with IVS3-48C/C. The FECH activity with IVS3-48C/C was only 38% of that with IVS3-48T/T. Then, we compared the FECH activities in EPP patients with those in healthy controls with IVS3-48C/C, C/T and T/T. As shown in Fig. 2, the activities in EPP patients relative to those of the controls were divided into three groups, which corresponded to 15, 35 and 64% of the controls, and these were dependent on the three genotypes. Various investigators have found that the FECH activities in EPP patients vary widely (8–45%), compared with those in controls [1, 9]. Some researchers reported that EPP seemed to exhibit

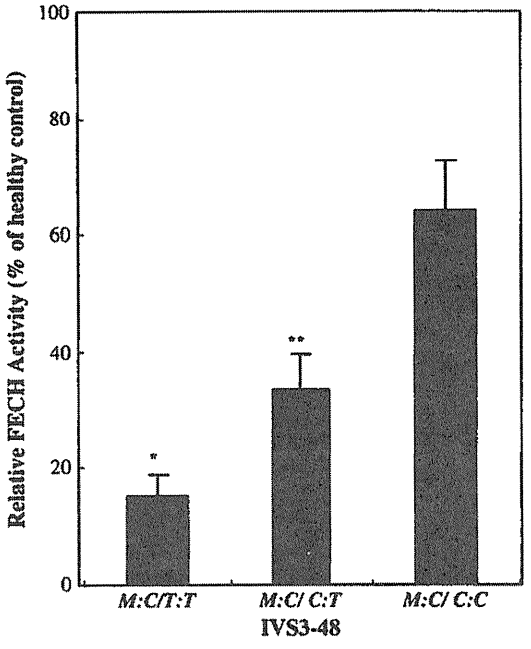

Fig. 2 The FECH activity in EPP patients relative to that of healthy controls with IVS3-48C/T transition. The FECH activity in peripheral blood lymphocytes of patients with EPP (*M:C*) was measured by comparison with that of controls with IVS3-48T/T (*T:T*) ($n = 4$), IVS3-48C/T (*C:T*) ($n = 6$) and IVS3-48C/C (*C:C*) ($n = 5$) of the gene. * $P < 0.01$, *M:C/T:T* versus *M:C/C:C*; ** $P < 0.01$, *M:C/C:T* versus *M:C/C:C*

autosomal recessive inheritance owing to the low enzyme activity [10]. We now demonstrate that this variation is derived from the three different genotypes of the *FECH* gene. Thus, heterozygotes with the low expression allele (IVS3-48C) in combination with a null allele would produce a small amount of FECH when compared with the normal group. Similarly, a low expression allele combined with a missense allele could explain the weak FECH activity observed in patients with EPP. Conversely, the FECH activities in healthy controls varied, the level of the relative FECH activities in EPP patients differed, depending on the different activities from the IVS3-48 genotypes of the *FECH* gene among controls. To estimate the frequency of IVS3-48C/T transition of the *FECH* gene in the Japanese population, analysis by single-strand conformation polymorphism (SSCP) using GeneGel Excel 12, 5/24 kit (GE Bioscience, Buckinghamshire, UK) was carried out with the genomic DNA of healthy volunteers. Of the 148 Japanese examined, the genotype with IVS3-48C/C was found in 32 (22%), IVS3-48C/T was in 68 (46%) and IVS3-48T/T was in 48 (32%). Thus, over half of the subjects have IVS3-48C. This value is similar to those reported for Asian people [2, 7]. Given that 10% of Caucasians have IVS3-48C, Asian people including Japanese face a higher risk of EPP. Although the reduced FECH activity is an important factor to diagnose EPP, it is difficult to evaluate EPP by FECH activity because of the high frequency of healthy controls with IVS3-48C in Asian populations.

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Mutations in the ribosomal protein genes in Japanese patients with Diamond-Blackfan anemia

Yuki Konno,[1] Tsutomu Toki,[1] Satoru Tandai,[1] Gang Xu,[1] RuNan Wang,[1] Kiminori Terui,[1] Shouichi Ohga,[2] Toshiro Hara,[2] Asahito Hama,[3] Seiji Kojima,[3] Daiichiro Hasegawa,[4] Yoshiyuki Kosaka,[4] Ryu Yanagisawa,[5] Kenichi Koike,[5] Rie Kanai,[6] Tsuyoshi Imai,[7] Teruaki Hongo,[8] Myoung-Ja Park,[9] Kanji Sugita,[10] and Etsuro Ito[1]

[1]Department of Pediatrics, Hirosaki University Graduate School of Medicine, Hirosaki; [2]Department of Pediatrics, Graduate School of Medical Sciences, Kyushu University, Fukuoka; [3]Department of Pediatrics, Nagoya University Graduate School of Medicine, Nagoya; [4]Department of Hematology and Oncology, Hyogo Children's Hospital, Kobe; [5]Department of Pediatrics, Shinshu University School of Medicine, Matsumoto; [6]Department of Pediatrics, Shimane University Faculty of Medicine, Izumo; [7]Department of Pediatrics, Otsu Red-Cross Hospital, Otsu; [8]Department of Pediatrics, Iwata City Hospital, Iwata; [9]Department of Hematology and Oncology, Gunma Children's Medical Center, Gunma, and [10]Department of Pediatrics, School of Medicine, University of Yamanashi, Yamanashi, Japan

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Correspondence: Etsuro Ito, M.D., Ph.D., Department of Pediatrics, Hirosaki University Graduate School of Medicine,5 Zaifucho, Hirosaki, Aomori, 036-8562 Japan. E-mail: eturou@cc.hirosaki-u.ac.jp

ABSTRACT

Background

Diamond-Blackfan anemia is a rare, clinically heterogeneous, congenital red cell aplasia: 40% of patients have congenital abnormalities. Recent studies have shown that in western countries, the disease is associated with heterozygous mutations in the ribosomal protein (RP) genes in about 50% of patients. There have been no studies to determine the incidence of these mutations in Asian patients with Diamond-Blackfan anemia.

Design and Methods

We screened 49 Japanese patients with Diamond-Blackfan anemia (45 probands) for mutations in the six known genes associated with Diamond-Blackfan anemia: *RPS19*, *RPS24*, *RPS17*, *RPL5*, *RPL11*, and *RPL35A*. *RPS14* was also examined due to its implied involvement in 5q- syndrome.

Results

Mutations in *RPS19*, *RPL5*, *RPL11* and *RPS17* were identified in five, four, two and one of the probands, respectively. In total, 12 (27%) of the Japanese Diamond-Blackfan anemia patients had mutations in ribosomal protein genes. No mutations were detected in *RPS14*, *RPS24* or *RPL35A*. All patients with *RPS19* and *RPL5* mutations had physical abnormalities. Remarkably, cleft palate was seen in two patients with *RPL5* mutations, and thumb anomalies were seen in six patients with an *RPS19* or *RPL5* mutation. In contrast, a small-for-date phenotype was seen in five patients without an *RPL5* mutation.

Conclusions

We observed a slightly lower frequency of mutations in the ribosomal protein genes in patients with Diamond-Blackfan anemia compared to the frequency reported in western countries. Genotype-phenotype data suggest an association between anomalies and *RPS19* mutations, and a negative association between small-for-date phenotype and *RPL5* mutations.

Key words: protein genes, Diamond-Blackfan anemia, *RPL5* mutation.

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Introduction

Diamond-Blackfan anemia (DBA, MIM#105650) is a congenital, inherited bone marrow failure syndrome, characterized by normochromic macrocytic anemia, reticulocytopenia, and absence or insufficiency of erythroid precursors in normocellular bone marrow.[1] DBA was first reported by Josephs in 1936 and defined as a distinct clinical entity 2 years later by Diamond and Blackfan. Recent investigations have shown that the cellular defect in DBA fibroblasts is primarily caused by reduced proliferation and a prolonged cell cycle corresponding to the bone marrow characteristics of DBA.[2] DBA is a rare disease with a frequency of two to seven cases per million live births and has no ethnic or gender predilection.[1]

Approximately 90% of affected patients typically present in infancy or early childhood, although patients with a 'non-classical', mild phenotype are diagnosed later in life.[3,4] Macrocytic anemia is a prominent feature of DBA, but the disease is also characterized by growth retardation and congenital anomalies, including craniofacial, upper limb/hand, cardiac, and genitourinary malformations that are present in approximately half of the patients.[3-5] In addition, DBA patients have a predisposition to malignancies including acute myeloid leukemia, myelodysplastic syndrome, and osteogenic sarcoma.[3] The diagnosis of DBA is often difficult because incomplete phenotypes and wide variability of clinical expression are present.[4-6] The central hematopoietic defect is enhanced sensitivity of hematopoietic progenitors to apoptosis along with evidence of stress erythropoiesis, including elevations in fetal hemoglobin and mean red cell volume.[2] The majority of patients have an increase in erythrocyte adenosine deaminase activity.[7]

Proteins are universally synthesized in ribosomes. This macromolecular ribonucleoprotein machinery consists of two subunits: one small and one large. The mammalian ribosome comprises four RNA and 80 ribosomal proteins.[8] The first genetic anomaly identified in DBA involves the *RPS19* gene, which is mutated in approximately 25% of DBA patients. This gene is located at chromosome 19q13.2 and encodes a protein belonging to the small subunit of the ribosome.[9,10] Haploinsufficiency of the *RPS19* gene product has been demonstrated in a subset of cases[11] and appears to be sufficient to cause DBA. The RPS19 protein plays an important role in 18S rRNA maturation and small ribosomal subunit synthesis in human cells.[12,13] Deficiency of RPS19 leads to increased apoptosis in hematopoietic cell lines and bone marrow cells. Suppression of *RPS19* inhibits cell proliferation and early erythroid differentiation but not late erythroid maturation in RPS19-deficient DBA cell lines.[14]

Mutations in two other genes, *RPS24* and *RPS17*, encoding proteins of the small ribosomal subunits have been found in approximately 2% of patients.[15,16] Furthermore, mutations in genes encoding large ribosomal subunit-associated proteins, *RPL5*, *RPL11* and *RPL35A*, have been reported in 9% to 21.4%, 6.5% to 7.1%, and 3.3% of patients, respectively.[17-19] To date, approximately 50% of DBA patients in western countries have been found to have a single heterozygous mutation in a gene encoding a ribosomal protein.[1,3] These findings imply that DBA is a disorder of ribosome biogenesis and/or function. However, there have been no studies of the incidences of these mutations in Asian DBA patients.

In this study, we screened 49 Japanese DBA patients (45 probands) for mutations of the six known DBA genes and *RPS14*, which has been implicated in the 5q- syndrome, a subtype of myelodysplastic syndrome characterized by a defect in erythroid differentiation.[20]

Design and Methods

Patients

Forty-nine patients were studied in order to define the frequency and type of mutations of ribosomal protein genes associated with DBA in Japan. Eight patients were from families with more than one affected member, whereas 41 were from families with only one affected patient. The diagnosis of DBA was based on the criteria of normochromic, often macrocytic anemia; reticulocytopenia; a low number or lack of erythroid precursors in bone marrow; and, in some patients, congenital malformations, without known causes of single cytopenia including acquired or congenital infection, transient erythroblastopenia of childhood, metabolic disorders, malignancies, or autoimmune diseases. All clinical samples were obtained with informed consent from 28 pediatric and/or hematology departments throughout Japan. Additional information was obtained by a standardized questionnaire including information on birth history, age of onset or diagnosis, family history, physical examination (especially regarding malformations), hematologic data, response to therapeutic procedures, and prognosis. This study was approved by the Ethics Committee of Hirosaki University Graduate School of Medicine.

Ribosomal protein gene analysis

DNA was extracted from peripheral blood using a standard proteinase K, phenol and chloroform protocol.[21] A polymerase chain reaction (PCR) was used to amplify fragments from genomic DNA using primer sets designed to amplify the coding exons and exon/intron boundaries of the *RPS19*, *RPS17*, *RPS24*, *RPS14*, *RPL5*, *RPL11* and *RPL35A*. PCR products were directly sequenced in the forward and/or reverse direction using the ABI PRISM BigDye Terminator Cycle Sequencing Ready Reaction Kit (Applied Biosystems, Tokyo, Japan) on an ABI PRISM 310 Genetic Analyzer (Applied Biosystems, Foster City, CA, USA). *RPS19* was analyzed by determining the genomic DNA sequence of the non-coding first exon, with flanking regions, and the 450-base pair (bp) sequence upstream of the first exon (5'UTR) for each DNA sample, as previously described.[5]

To clarify the sequence of heterozygous insertion/deletion sequence variations, the respective PCR products were cloned into a TA pCR 2.1 vector (Invitrogen, Carlsbad, CA, USA) and their sequences were confirmed.

Genotype-phenotype correlations and statistical analysis

Physical abnormalities in the Japanese DBA patients were evaluated from a viewpoint of correlations with genotype. Although growth retardation can be modified by several factors such as steroid therapy, chronic anemia, and iron overload, the retardation was considered pathognomonic for DBA if it was marked, being below -3 standard deviations (SD). Response to treatment is usually seen within 1 month of treatment in DBA, but a prediction of response has not been reported previously.[1,3] We, therefore, also examined the correlation between genotype and response to the first round of steroid therapy. Associations between two groups of variables were assessed with Fisher's exact test. All tests were two-sided and P levels less than 0.05 were considered statistically significant. Data were analyzed with SPSS 11.0J software (SPSS Inc., Chicago, IL, USA).

Results

Patients' characteristics

Overall, 49 patients (45 probands) were available for analysis. The male to female ratio was 1:1.2. Forty-one index cases were classified as sporadic without unexplained anemia in first-degree relatives, while the remaining eight patients were from four families. All patients were Japanese except two cases: case 10 was Chinese and case 23 was a Brazilian of Japanese extraction. Case 15 had a Filipina mother and a Japanese father.

Genetics

RPS19

Five different mutations were detected in five probands out of 45 families (11%) (Table 1). The median age at presentation of the index cases with *RPS19* mutations was 1 month (range, 0 to 2 months). There appears to be a lower percentage of *RPS19* mutations in Japanese DBA patients than in patients from western countries. All mutations were in the coding region of the gene. Missense mutations resulting in amino acid substitutions were noted in four index cases. The three mutations, p.R62Q in case 30, p.R62W in case 44 and p.0 in case 43, have been reported in seven, ten and two families, respectively,[6,10,11,22-26] whereas one mutation, p.G95V in case 25, was novel, and could not be found in the Single Polymorphism Database (dbSNP at *www.ncbi.nlm.nih.gov/SNP*). Furthermore, the mutation was not observed in DNA from 50 normal individuals. An insertion of one nucleotide was found in one case (case 28), resulting in a novel frameshift mutation.

RPL5 and RPL11

The human *RPL5* gene consists of eight exons and is located on chromosome 1. Four novel mutations were found among the 45 probands (9%) (Table 1). The median age at presentation of the index cases with *RPL5* mutations was 10 months. A deletion of two nucleotides was found in case 10, and an insertion of one nucleotide was found in case 65, each affecting the reading frame. Two cases (cases 41 and 55) had point mutations that resulted in a loss of the translation initiation codon.

The human *RPL11* gene, which consists of six exons, is also located on chromosome 1. All exons and exon/intron boundaries were PCR-amplified and sequenced in DBA patients who were negative for mutations in *RPS19* and *RPL5*. Two mutations (4%) were found, and they were diagnosed at 18 and 20 months old, respectively (Table 1). A deletion of two nucleotides was found in case 9, and a deletion of one nucleotide was found in case 23, in each patient leading to a shift in the reading frame and the introduction of a premature stop codon.

RPS17

The *RPS17* gene is located on chromosome 15, and consists of five exons. *RPS17* mutations are rare and have been reported in only two patients with DBA. A novel one-nucleotide deletion in *RPS17* was identified in one patient (2%), resulting in the introduction of a premature stop codon (Table 1). The patient with the *RPS17* mutation (case 56) was born to healthy non-consanguineous parents and diagnosed as having DBA at the age of 1 month. He responded to the initial steroid treatment, and had a course of steroid-dependent therapy. No physical anomalies were seen in this patient.

RPL35A, RPS24 and RPS14

Mutations in *RPS24* and *RPL35A* are rare and have been reported in only eight and six patients with DBA, respectively. DBA patients were screened for *RPS24* and *RPL35A*, in addition to *RPS14*, which is implicated in the 5q- syndrome. No mutations were detected in *RPS24*, *RPL35A* or *RPS14* in Japanese DBA patients.

In total, sequence changes were found in four out of seven screened ribosomal protein genes (Table 2). Mutations in *RPS19*, *RPS17*, *RPL5*, and *RPL11* were detected in 11%, 2%, 9%, and 4% of the probands, respectively. The frequency of ribosomal protein gene mutations in Japanese DBA patients was 27%.

Genotype-phenotype correlations: congenital anomalies

The patients' characteristics are summarized in Table 3.

Table 1. Mutations identified in *RPS19*, *RPL5*, *RPL11*, and *RPS17* in Japanese DBA patients.

Patients (gender)	Inheritance	Age at diagnosis	Mutation	Predicted amino acid change
Mutations in the *RPS19* gene				
43 proband (F)	Sporadic	0 D	Exon2:g.3G>A	p.0
28 proband (M)	Sporadic	6 D	Exon3:g.130_131insA	E44fsX50
44 proband (F)	Sporadic	1 M	Exon4:g.184C>T	R62W
30 proband (F)	Familial	1 M	Exon4:g.185G>A	R62Q
30 father (M)	Familial	0 M	Exon4:g.185G>A	R62Q
25 proband (M)	Sporadic	2 M	Exon4:g.284G>T	G95V
Mutations in the *RPL5* gene				
10 proband (F)	Sporadic	0 M	Exon5:g.473_474delAA	K158fsX183
41 proband (F)	Sporadic	1 Y	Exon1:g.3G>T	p.0
55 proband (F)	Sporadic	3 Y	Exon1:g.3G>A	p.0
65 proband (F)	Sporadic	4 M	Exon2:g.37_38insT	F13fsX14
Mutations in the *RPL11* gene				
9 proband (F)	Sporadic	1 Y 10 M	Exon2:g.58_59delCT	L20fsX53
23 proband (M)	sporadic	1 Y 6 M	Exon5:g.460delA	R154fsX189
Mutations in the *RPS17* gene				
56 proband (F)	Sporadic	1 M	Exon2:g.26delT	V9fsX17

Anomalies associated with DBA were found in 27 patients (55%). Sixteen had two or more malformations (33%). All six patients with an *RPS19* mutation had physical anomalies, and three of them had multiple anomalies. In contrast, clinical data from European and American DBA patients showed that the frequency of malformations was 31% in patients with *RPS19* mutations, which is not significantly different from that of the entire DBA population.[26] *RPS19* mutations are characterized by a wide variability of phenotypic expression.[26] A mutation is frequently associated with various degrees of anemia, different responses to treatment, and dissimilar malformations. Even various family members having the same mutation in *RPS19* present with different clinical expressions. Cases 30, 44 and 43 harbored the same *RPS19* mutations reported in multicase families (p.R62Q, p.R62W, p.0).[6,10,11,22-27] Comparable to previous observations, no consistent clinical features were found in patients from different families displaying mutations in *RPS19*. For example, the father of case 30 harboring the same mutation had no finger anomalies, although case 30 had syndactyly and thumb polydactyly.

Consistent with reports that patients with *RPL5* and *RPL11* mutations are at high risk of developing malformations,[17,18] all four patients with *RPL5* mutations had physical anomalies. Furthermore, three of them had multiple physical anomalies, particularly case 41, who had very severe congenital heart disease (Table 3). One of two patients with *RPL11* mutations had physical anomalies. In contrast, of the 36 patients with no mutations, physical anomalies were seen in 16 (44%).

Nine patients had craniofacial anomalies. Of these, two had *RPL5* mutations, while the remaining patients had no mutations. Gazda *et al.* suggested an association between *RPL5/RPL11* mutation and cleft lip and/or palate.[17] Data in the Diamond-Blackfan Anemia Registry (DBAR) of North America also suggest that the DBA phenotype associated with cleft lip/palate is caused by non-*RPS19* mutations.[4] In our cohort, the frequency of cleft palate was significantly different between *RPL5*-mutated and *RPL5* non-mutated groups ($P<0.05$): cleft palate was seen in three patients, two of whom had *RPL5* mutations while the other patient belonged to the *RPL5* non-mutated group.

Thumb anomalies were seen in six patients, four of whom had *RPS19* mutations while two had *RPL5* mutations. There was a statistically significant difference in the frequency of thumb anomalies between *RPS19*-mutated and *RPS19* non-mutated groups ($P<0.05$). Flat thenar was seen in one patient with an *RPL5* mutation. In contrast to previous reports on patients with *RPL11* mutations, thumb anomalies were not found in our patients with these mutations.

A small-for-date phenotype was seen in seven patients (14%): one had an *RPS19* mutation, one had an *RPL11* mutation, and the four others had no mutations. None of the patients with *RPL5* mutations was born small-for-date.

Genotype-phenotype correlations: therapeutic response

Corticosteroids and transfusions are the mainstays of DBA treatment.[1,3] Of 45 patients evaluable for first treatment response, 73% responded to steroid therapy, 8% did not respond and 16% were never treated with steroids. The proportions of patients who responded to the first steroid treatment were 5/5 (*RPS19*), 2/3 (*RPL5*), 1/2 (*RPL11*), 1/1 (*RPS17*), and 22/27 (no mutation). There were no significant differences in the response rates among these patients.

Sixty-nine percent of patients received red blood cell transfusions. Of 48 patients available for therapy in follow-up, 8 patients (17%) were transfusion-dependent, 18 patients (37%) were steroid-dependent, and 18 patients (37%) were transfusion-independent with no other treatment. Three patients received bone marrow transplants and were alive and well (Table 3). A malignancy was detected in one case (case 50, proband), who developed a myelodysplastic syndrome 1 year after the diagnosis of DBA.

Table 2. Summary of sequence changes in seven ribosomal protein genes identified in Japanese DBA patients.

Gene symbol	N. of tested DNA samples from unrelated probands	N. of probands with mutations	N. of subjects with mutations	Mutation types
RPS19	45	5 (11%)	6	missense, loss of 1st methionine, small insertion
RPL5	45	4 (9%)	4	loss of 1st methionine, small deletion, small insertion
RPL11	34	2 (4%)	2	small deletion
RPS17	45	1 (2%)	1	small deletion

Discussion

This is the first report of an investigation of DBA patients in Japan. Twelve types of mutations were detected in four ribosomal protein genes. These mutations occurred in 27% of Japanese DBA patients. Mutations in *RPS19*, which have been found in 25% of patients in western countries,[26] were detected in only five of 45 probands (11%) in Japan, and two of these mutations were unique. Novel mutations in *RPL5* (four probands; 9%), *RPL11* (two probands; 4%) and *RPS17* (one proband; 2%) were identified. The frequencies of mutations in *RPL5*, *RPL11* and *RPS17* were very similar to those in western countries.[16-19] These results may suggest that a lower incidence of mutations in ribosomal protein genes in Japanese patients with DBA is due to a lower incidence of *RPS19* mutations, although we might have missed large deletions or re-arrangements in this study.

Physical abnormalities and growth retardation were detected in 55% of the Japanese DBA patients, consistent with previous reports from western countries.[4-6] Recent studies suggest that patients with *RPL5* mutation are more likely to have physical malformations including craniofacial, thumb, and heart anomalies.[17,18] Remarkably, patients with *RPL5* mutations tend to have cleft lip and/or palate or cleft soft palate, isolated or in combination with other physical abnormalities.[17,18] We found that three of four patients with *RPL5* mutations had multiple physical malformations, and two had cleft palate, whereas only one patient without an *RPL5* mutation had cleft palate. In the general population, 0.1% to 0.2% of children are born with cleft lip and/or palate.[28] Our data, and those from previous findings, suggest that *PRL5* mutations are associ-

Table 3. Characteristics of Japanese DBA patients.

Patient	Malformation status	Response to first steroid therapy	Present therapy
Patients with mutation of *RPS19*			
25 proband	Thumb polydactyly, growth retardation (-2.0SD), etc.	ND	ND
28 proband	Thumb polydactyly, CHD, etc.	Response	Steroid-dependent
30 proband	Thumb polydactyly, syndactyly, growth retardation (-3.4SD)	Response	Steroid-dependent
30 father	Growth retardation (-3.6SD)	NA	CR
43 proband	Thumb polydactyly	Response	Steroid-dependent
44 proband	SFD	Response	CR
Patients with mutation of *RPL5*			
10 proband	Flat thenar, cleft palate, CHD, etc.	Poor	Transfusion-dependent
41 proband	Craniofacial abnormalities, cleft palate, CHD, etc.	ND	Transfusion-dependent
55 proband	Thumb polydactyly	Response	Steroid-dependent
65 proband	Growth retardation (-3.0SD)	Response	Steroid
Patients with mutation of *RPL11*			
9 proband	CHD, SFD, etc.	Response	CR
23 proband	None	Poor	Steroid-dependent
Patient with mutation of *RPS17*			
56 proband	None	Response	Steroid-dependent
Patients without mutation of seven RP genes			
1 proband	Growth retardation (-4.0SD)	Response	CR
1 daughter	None	Response	CR
3 proband	Growth retardation (-3.6SD)	Response	Steroid-dependent
4 proband	Craniofacial abnormalities, SFD, short stature, webbed neck	Response	Steroid-dependent
5 proband	None	Response	CR
6 proband	Cleft palate, SFD, etc.	Poor	BMT
7 proband	Craniofacial abnormalities, SFD, growth retardation, etc.	Response	CR
8 proband	Growth retardation, webbed neck	Response	Steroid-depndent
13 proband	None	NA	CyA, BMT
14 proband	None	Response	CR
15 proband	None	Response	Transfusion-dependent
20 proband	Craniofacial abnormalities, CHD, etc.	Rsponse	Transfusion-dependent
21 proband	None	Response	Steroid-dependent
22 proband	None	Response	CR
24 proband	Growth retardation (-4.0SD)	Response	Steroid-dependent
26 proband	Growth retardation (-4.1SD), craniofacial abnormalities, etc.	Response	Transfusion-dependent
33 proband	None	Response	BMT
36 proband	Hypospadias, cryptorchidism	Response	Steroid-dependent
36 cousin	None	Response	Steroid-dependent
37 proband	Hypospadias, cryptorchidism	ND	CR
42 proband	None	Response	CR
45 proband	Craniofacial abnormalities, growth retardation, etc.	Poor	Transfusion-dependent
48 proband	Fetal hydrops	ND	CR
49 proband	None	Response	Steroid-dependent
50 proband	None	Response	Steroid-dependent, CBT (due to MDS)
50 sister	None	Response	Steroid-dependent
51proband	None	Poor	CR
54 proband	None	ND	Transfusion-dependent
59 proband	None	ND	Transfusion
60 proband	SFD	ND	Transfusion
61 proband	None	Response	Cyclosporine
62 proband	CHD, SFD, growth retardation (-3.1SD)	Response	Steroid-dependent
63 proband	Craniofacial abnormalities, growth retardation (-7.5SD)	Response	Steroid-dependet
64 proband	None	Response	Steroid-depndent
66 proband	None	NA	Transfusion-dependent
67 proband	None	NA	NA

*ND: not done; NA: not available; SFD: small-for-date; CHD: congenital heart disease; MDS: myelodysplastic syndrome; BMT: bone marrow transplantation; CBT: cord blood stem cell transplantation; CR: complete remission. * RPS19, RPS24, RPS17, RPS14, RPL5, RPL11, RPL35A.*

ated with multiple physical abnormalities, especially cleft lip and/or palate.

Cmejla *et al.* reported that 87.5% of *RPL5*-mutated patients were born small-for-date, whereas only 42.9% of *RPS19*-mutated patients were born small-for-date.[18] However, in our series, the small-for-date phenotype was seen in seven patients, and all of them were *RPL5*-non-mutated patients. Our data suggest that *RPL5* mutations in Japanese DBA patients have no relevance to the small-for-date phenotype, which may be a unique characteristic of Japanese DBA.

According to recent studies, the frequency of malformation, particularly thumb anomalies, in *RPS19*-mutated patients, was relatively low compared to that in *RPL5*- or *RPL11*-mutated patients.[22-24,29] In Italian DBA patients, the risk of malformation was 7-fold higher in *RPL5*-mutated patients than in *RPS19*-mutated patients.[29] In contrast, all of the Japanese DBA patients with *RPS19* mutations had one or more malformations. The frequency of thumb anomalies was significantly higher in patients with *RPS19* mutations, as well as in patients with *RPL5* mutations, compared to in the other groups of patients.

Although steroid therapy is one of the established treatments for DBA, the mechanism of action is unknown and reliable prediction of response to initial steroid therapy is not available.[1,3] *RPS19* mutation status has not been predictive of response in any series.[3] In our cohort, responsiveness to first steroid therapy in Japanese DBA patients was as good as that reported in western populations.[1,3] In this study, no significant differences in response to initial steroid therapy were found between *RPS19*-mutated and *RPS19*-non-mutated groups, or between the groups with *RPS19* mutations and other ribosomal protein gene mutations.

In summary, we found that heterozygous mutations in *RPS19*, *RPL5*, *RPL11* or *RPS17* were present in 27% of Japanese DBA patients. No mutations were detected in *RPS14*, *RPS24* or *RPL35A*. We observed a slightly lower frequency of mutations in ribosomal protein genes in our cohort of Japanese DBA patients than the frequencies reported previously from western countries, although the data from both populations are based on relatively low numbers of patients and values showing significant differences between populations are lacking. Our data suggest an association between *RPL5* mutation and malformations, especially cleft palate, and between *RPS19* mutation and malformations, particularly thumb anomalies. This study also suggests that no association exists between *RPL5* mutations and the small-for-date phenotype or between *RPS19* mutations and non-responsiveness to initial steroid therapy in Japanese DBA patients.

Authorship and Disclosures

EI was the principal investigator and takes primary responsibility for the paper. YK, TT, ST, GX, RNW, KT, and SO performed the laboratory work for this study. SO, TH, AH, SK, DH, YK, RY, KK, RK, TI, TH, MHP, and KS enrolled the patients. EI and YK wrote the paper.

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