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To the editor:

Peripheral blood stem cells versus bone marrow in pediatric unrelated donor stem cell transplantation

The relative benefits and risks of peripheral blood stem cells (PBSCs) versus bone marrow (BM) for allogeneic hematopoietic stem cell transplantation (SCT) are still a matter of highly controversial debates.[1-3] The first randomized study comparing the 2 stem cell sources in unrelated donor SCT recently documented comparable overall and event-free survival, but indicated a higher risk for chronic graft-versus-host disease (GVHD) with PBSCs.[4] Only a few pediatric patients were included in this study even though the long-term sequelae of chronic GVHD are of particular concern in this patient group.

We retrospectively compared the long-term outcome of contemporaneous unrelated donor SCT in 220 children transplanted with BM (n = 102) or PBSCs (n = 118) for hematologic malignancies and reported to the German/Austrian pediatric registry for SCT. All patients had received myeloablative conditioning followed by unmanipulated SCT from HLA-matched unrelated donors. The PBSC and BM groups were comparable with regard to patient and donor age, sex, cytomegalovirus (CMV) serostatus, disease status at transplantation, GVHD prophylaxis, growth factor use, and degree of HLA matching. The groups differed with regard to disease category with slightly more myelodysplastic syndrome patients ($P = .02$) and a higher CD34-cell dose ($P = .001$) in the PBSC group.

Neutrophil and platelet engraftment were achieved significantly faster after PBSC than BM transplantation (Figure 1A-B). In this entirely pediatric cohort, the incidence of clinically relevant grade II-IV acute GVHD (Figure 1C) did not differ. Most importantly, the incidence of chronic GVHD (PBSCs vs BM: 35% vs 33%, respectively; $P = .9$) and extensive chronic GVHD (Figure 1D) proved low and was virtually identical in the 2 groups. With a median follow-up time of 3 years, overall survival (PBSCs vs BM: 50% ± 5% vs 46% ± 6%, respectively; $P = .63$) and event-free survival (PBSCs vs BM: 45% ± 5% vs 44% ± 6%, respectively; $P = .59$) were comparable (Figure 1E-F). In multivariable analysis, taking into account all parameters with $P < .2$ in univariate analysis, the only significant independent risk factor for treatment failure was advanced disease status at the time of transplantation (relative risk = 2.4, 95% confidence interval, 1.5-3.8; $P = .001$). In contrast, stem cell source (PBSCs vs BM) had no effect (relative risk = 1.1, 95% confidence interval, 0.7-1.6; $P = .8$).

Our registry-based analysis provides evidence that in pediatric recipients of HLA-matched unrelated-donor transplantation with consistent antithymocyte globulin (ATG) use during conditioning, transplantation with PBSCs and BM results in comparable clinical outcomes without detectable differences in the risk of acute or, more importantly, chronic GVHD. Consistent with a recent study underscoring the role of ATG for the prevention of acute and chronic GVHD,[5] the use of ATG in 96% of our transplantation procedures compared with only 27% in the above-mentioned randomized study by Anasetti et al[4] might be one of the key factors responsible for the overall low and comparable incidence of

A Novel Mutation of Ribosomal Protein S10 Gene in a Japanese Patient With Diamond-Blackfan Anemia

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Summary: Diamond-Blackfan anemia (DBA) is an inherited bone marrow disease. The condition is characterized by anemia that usually presents during infancy or early childhood and congenital malformation. Several reports show that DBA is associated with mutations in the ribosomal protein (RP) genes, *RPS19*, *RPS24*, *RPS17*, *RPL35A*, *RPL5*, *RPL11*, and *RPS7*. Recently, 5 and 12 patients with mutations in *RPS10* and *RPS26*, respectively, were identified in a cohort of 117 DBA probands. Therefore, we screened the DBA patients who were negative for mutations in these DBA genes for mutations in *RPS10* and *RPS26*. The present case report describes the identification of the first Japanese DBA patient with a novel mutation in *RPS10*.

Key Words: Diamond-Blackfan anemia, ribosomal protein genes, mutation in *RPS10*

(*J Pediatr Hematol Oncol* 2012;34:293–295)

Diamond-Blackfan anemia (DBA) is an inherited bone marrow disease. The condition is characterized by anemia that usually presents during infancy or early childhood, congenital malformation, and an increased incidence of cancer.[1–3] In 1999, it was reported that DBA is associated with mutations in the ribosomal protein (RP) gene, *RPS19*.[4] This mutation was identified in 25% of DBA probands and prompted the search for other RP gene mutations. Subsequently, DBA patients with mutations in *RPS24*, *RPS17*, *RPL35A*, *RPL5*, *RPL11*, and *RPS7* were reported, suggesting that DBA is a disorder of ribosomal biogenesis and/or function.[5–7] Recently, Doherty et al[8] reported 3 distinct mutations of the *RPS10* in 5 patients from a cohort of 117 DBA probands. Therefore, we screened the Japanese DBA patients who were negative for mutations in these RP genes for mutations in *RPS10* and *RPS26*. Here, we report the first Japanese DBA patient with a novel mutation in *RPS10*.

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CASE REPORT

A 6-year-old boy was referred to our hospital with anemia with no other significant cytopenia. He was an only child with no family history of anemia. He has no congenital malformations described in "classical DBA," apart from bilateral lymphangioma of the foot. His white blood cell count was 4.3×10^9/L, the erythrocyte count was 2540×10^9/L, the hematocrit was 24.6%, hemoglobin concentration was 8.3 g/dL, the mean corpuscular volume was 96.9 fl, the mean corpuscular hemoglobin was 32.7 pg, the platelet count was 278×10^9/L, and the reticulocyte count was 1.5%. The fetal hemoglobin was 1.4%. The serum iron was 93 µg/dL, the serum unsaturated iron-binding capacity was 184 µg/dL, and the serum ferritin was 9 ng/mL. The serum vitamin B12 was 850 pg/mL and the serum folic acid was 6.8 ng/mL. The serum aspartate aminotransferase was 17 U/mL, the alanine aminotransferase was 10 U/mL, and the lactate dehydrogenase was 201 U/mL. The erythropoietin level was 1170 mU/L. The serum total bilirubin was 0.5 mg/dL. The direct and indirect Coombs' tests were negative. The anti-B19 parvovirus immunoglobulin M and immunoglobulin G antibodies were negative. Bone marrow aspiration showed that the cellularity was slightly hypoplastic (78500/µL), with a paucity of erythroid cells (16.8%; macrocytic-basophilic erythroblasts, 0.4%, noromocytic-basophilic erythrobalasts, 1.2%, noromocytic-polychromic erythroblasts, 10.4%, normocytic-orthochromic erythroblasts, 4.8%), but the morphology was normal. It showed that myeloid cells (34.4%) have no abnormalities associated with myelodysplastic syndromes. Lymphoid cells (38%) and megakaryocytes were normal. Cytogenetic analysis showed no chromosomal abnormality. On the basis of these findings, DBA was diagnosed in this patient.[1] The patient responded to oral steroids but not to cyclosporine. A small dose of prednisolone (0.18 to 0.23 mg/kg/d) were given to maintain an erythrocyte count of 2500×10^9/L, a hemoglobin concentration of 8.0 g/dL, and his daily activities. The most distressing complication has been obesity. He has never received blood transfusion.

At 22 years of age, analysis of RP genes was performed. Informed consent was obtained according to the guidelines set out by Hirosaki University Graduate School of Medicine. Initially, the patient was screened for mutations in the 8 genes known to be associated with DBA, *RPS19*, *RPS24*, *RPS17*, *RPL5*, *RPL11*, *RPL35A*, *RPS10*, and *RPS26*, using high-resolution amplicon melting analysis. He was also screened for *RPS14* mutations, which are a causative gene for 5q-syndrome. The results showed a separated signal derived from the heteroduplex polymerase chain reaction product from the third exon of *RPS10*. Direct sequencing analysis of the polymerase chain reaction product and the cloned amplicon identified a heterozygous mutation (283_306delinsTGCC) (Fig. 1). This mutation resulted in a frameshift at codon 95 and a "stop" at codon 100 (Fig. 2).

DISCUSSION

Nine RP genes, *RPS19*, *RPS24*, *RPS17*, *RPL5*, *RPL11*, *RPL35A*, *RPS14*, *RPS10*, and *RPS26*, were screened in 64 Japanese probands with DBA. Screening identified 8, 6, and 3 patients with mutations in *RPS19*, *RPL5*, and *RPL11*, respectively, and a single patient each with a mutation in *RPS17*, *RPS10*, and *RPS26*[9] and

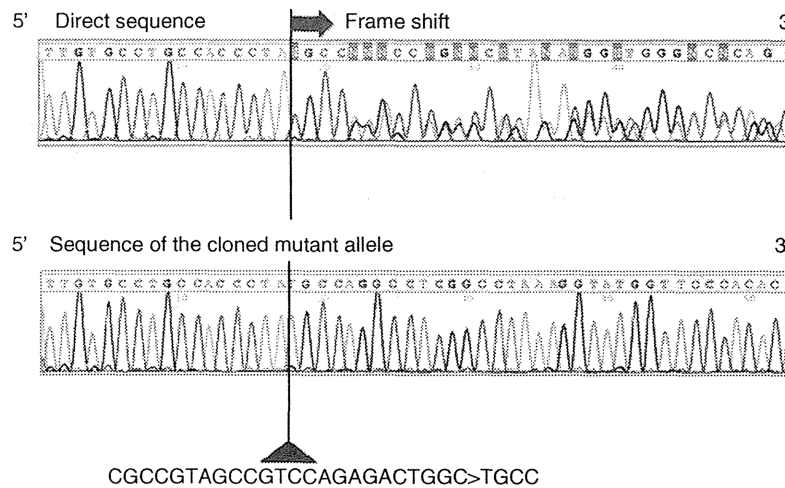

FIGURE 1. Sequence changes and frameshift in the *RPS10*. Direct sequencing showed a separated signal derived from the heteroduplex polymerase chain reaction product from the third exon of *RPS10*. Sequencing of the cloned mutant allele identified a heterozygous mutation (c.283_306delinsTGCC) and frameshift.

(unpublished data). In total, 20 (31.3%) of the Japanese DBA patients had mutations in RP genes. This is a slightly lower frequency than that reported in Western countries, although the data from both populations are based on relatively low numbers of patients, and data showing significant differences between populations are lacking.

The RPSP10 gene is located on chromosome 6 and contains 6 exons, with the start codon in exon 2. *RPS10* encodes a protein of 165 amino acids, which is a component of the 40S ribosomal subunit. To our knowledge, this is the first report of a Japanese DBA patient with a mutation in *RPS10*. The mutation (283_306delinsTGCC) results in a frameshift at codon 95 and the premature termination of codon 100. This novel mutation has not been reported in the literature. Doherty et al[8] identified 3 heterozygous sequence changes in *RPS10* in 5/117 probands, with no evidence of mutations in any of the known DBA genes. One sequence change was a missense mutation 3G > A (Met1 to Ile), which eliminates the start codon. The next downstream start codon is located at nucleotide position 61 to 63 and is predicted to start translation of a truncated protein. Another mutation was c.260_261insC, which results in a frameshift at codon 87 and a "stop" at codon 97. Three other probands contained a common nonsense mutation, c.337C > T, causing an Arg113 "stop." In our case, the mutation seems to be the result of both a deletion and an insertion. These mutations are very rare in DBA. To understand the mechanism of mutagenesis, we examined *RPS10* psuedogenes (*PRS10P1* to *RPS10P31*) to see if this mutation arose from interlocus gene conversion. However, we could find no evidence that the mutation arose due to gene conversion. The authors estimated that *RPS10* mutations were present in about 2.6% of the DBA population. Although more information is needed to estimate the incidence of *RPS10* mutations in Japanese DBA patients, the frequency of *RPS10* mutations in the Japanese population was similar to that in Western countries. All the *RPS10* mutations observed in patients with DBA, including our case, are nonsense or frameshift mutations. Nonsense and frameshift mutations are likely to be pathogenic in the majority of cases; however, determining the pathogenicity of a particular missense mutation may be difficult.

The RPS19 protein plays an important role in 18S rRNA maturation in both yeast and human cells.[10–13] Other

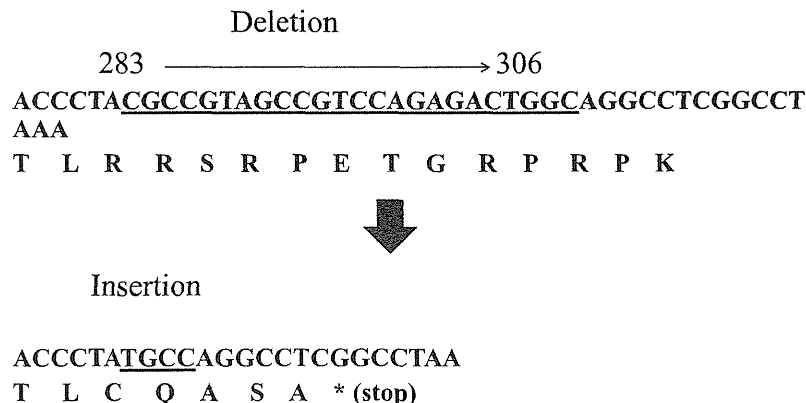

FIGURE 2. Deletion and insertion of this patient in *RSP10*. The c.283_306delinsTGCC mutation resulted in a frameshift at codon 95 and a "stop" at codon 100.

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studies demonstrate alterations of pre-RNA processing and small or large ribosomal subunit synthesis in human cells with *RPS24, RPS7, RPL35A, RPL5*, and *RPL11* deficiency.[14–16] Increased apoptosis has been demonstrated in hematopoietic cell lines and bone marrow cells deficient in RPS19 and RPL35A.[14,17,18] Imbalances in p53 family proteins have been suggested as a mechanism of abnormal embryogenesis and anemia in zebrafish upon perturbation of RPS19 expression.[19] Also, the DBA phenotype in mice was ameliorated by knockdown of p53.[20] We hope to use hematopoietic progenitor cells to investigate why mutations in *RPS10* affect erythropoiesis in DBA patients.

Patients with "classical DBA" fulfill all the major diagnostic criteria, including anemia presenting before the first birthday.[1] However, a definitive diagnosis of DBA is often difficult because of incomplete phenotypes and a wide variation in clinical expression. This particular patient presented with macrocytic anemia at 6 years of age, with no family history and none of the congenital anomalies described for "classical DBA." The identification of pathogenic mutations in *RPS10* provides a definitive diagnosis of DBA in this patient. Although the use of molecular diagnostic techniques is essential to establish a definitive diagnosis and research the cause of DBA, such a diagnosis in only obtained for 30% to 40% of patients. Therefore, it is important to identify all genes that cause DBA if we are to improve the efficiency of molecular diagnostic techniques and understand the pathogenesis of DBA.

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【第53回日本小児血液・がん学会学術集会】シンポジウム1：本邦における骨髄不全症候群の現況

先天性赤芽球癆（Diamond-Blackfan 貧血）の効果的診断法の確立に関する研究

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要　旨

Diamond-Blackfan 貧血（DBA）は，乳児期に発症する稀な先天性赤芽球癆である．約40% に種々の先天異常を合併する．欧米では約50% の DBA 患者にリボソームタンパク（RP）遺伝子の変異が認められるが，本邦の DBA 患者の RP 遺伝子変異の頻度は不明であった．我々は，本邦で発症した83例の DBA 患者（76例の発端者）の末梢血から DNA を抽出し，DBA で遺伝子変異が報告されている8遺伝子を解析した．その結果，*RPS19* 遺伝子変異が10例（8家系），*RPL5* 変異は6例（6家系），*RPL11* 変異は4例（4家系）で検出された．*RPS10*，*RPS17* と *RPS26* 変異は，それぞれ1例（1家系）に認められた．興味深いことに，*RPL5* 変異をもつ患者は6例中5例が身体的異常を合併し，そのうちの2例は口蓋裂を合併していた．次に，通常のシークエンス解析では片アレル欠失は検出できないため，定量的 PCR 法を用いた新規の片アレル欠失検出法を開発し，原因遺伝子が不明である27症例の解析を行った．その結果，片アレル欠失を7例（25.9%）に認めた．片アレル欠失は *RPS17*（3例），*RPS19*（2例），*RPL5*（1例），*RPL35a*（1例）であった．以上，本邦における DBA の発端者76例中28例（36.8%）に RP 遺伝子の変異を認めた．DBA の効果的診断法の確立のためには，さらに新規原因遺伝子の解明と信頼できるバイオマーカーの同定が必要である．

キーワード：Diamond-Blackfan 貧血，リボゾーム蛋白，RPS19，リボゾーム病
Key words: Diamond-Blackfan anemia, ribosomal protein, RPS19, ribosome, haploinsufficiency

I　はじめに

Diamond-Blackfan anemia（DBA）は，乳児期に発症する赤血球造血のみが障害される先天性の赤芽球癆である．骨髄は正形成であるが，赤血球系細胞のみが著減し，末梢血では網赤血球が減少し，大球性正色素性貧血を呈する．約30% の症例で様々な奇形を合併することが知られている．大頭，小頭などの頭部，顔部の異常が最も多く，上肢，眼，泌尿生殖器系，心臓の異常や低身長が見られる．ほとんどが散発例であるが，約10～20% の症例では家族歴があり，常染色体性優性あるいは劣性遺伝の形式をとる[1]．

1936年，Josephs により2例[2]，2年後には Diamond および Blackfan により congenital hypoplastic anemia として4例が報告[3]されて以来，この疾患の病因に関する様々な研究が行われてきた．造血微小環境や支持細胞よりむしろ赤血球系造血前駆細胞自体の分化増殖能になんらかの heterogenous な異常が存在する可能性が高いと推定されていたが，長らく病因は不明であった．1997年，原因遺伝子の一つが80個あるリボソームタンパクの一つである *PRS19* をコードする遺伝子であることが明らかにされた[4]．*RPS19* 遺伝子変異は約25% の DBA 患者に認められるが，最近 *RPS24*，*RPS17*，*RPL5*，*RPL11* および *RPL35a* の遺伝子変異が少数例の DBA で発見され，リボソームの異常に起因した新たな疾患「リボソーム病」の疾患概念が確立されつつある[5]．

II　DBA の原因遺伝子の発見

1938年，既に Diamond と Blackfan は，DBA の原因が先天的な造血システムの異常であると推定していた．しか

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し，DBA の原因は 19 番染色体上の最初の DBA 遺伝子が見つかるまでは大きな謎であった．Gustavsson らは，相互転座 46, XX, t(X;19)(p21;q13) を持つ DBA 患者を見出した[6]．19 番染色体の長腕上に DBA の原因遺伝子が存在する可能性が示唆されたため，DBA の 13 家系の連鎖解析を染色体 19q のマーカーを用いて行った．その結果，19q13 に強い連鎖を認め[7]，さらに 3 例の DBA 患者に 19q13 上の部分的に重なり合う microdeletion を見出した[8]．以上の結果より，DBA 病因遺伝子の遺伝子座が 19q13 に存在することが強く示唆されたため，上記の相互転座を持つ DBA 患者の DNA から，positional cloning の手法で原因遺伝子の同定が行われた．即ち，19q13 上の転座切断点を含む領域をクローニングし，切断点がリボソームタンパク遺伝子 *RPS19* の第 3 イントロンに存在することを明らかにした．さらに，40 例の DBA 症例を解析し，10 例に *RPS19* 遺伝子の変異を検出した[4]．その後，大規模なスクリーニングが行われ，172 家系の DBA 症例が解析され，約 25% の患者に *RPS19* 遺伝子の変異が認められた[9]．遺伝子の変異は，ミスセンス，ナンセンス，スプライスサイト，フレイムシフト変異と様々であったが，全てヘテロ変異であった．遺伝子変異は広く遺伝子全体に散らばっていたが，変異のホットスポットがコードン 52～62 の間（エクソン 3 とエクソン 4）に存在していた．しかし，遺伝子変異の性質と臨床症状との間には，はっきりした関係は認められなかった．その後，欧米を中心にさらに解析が進められ，約 20～25% の DBA 症例に *RPS19* の変異が見出された[10,11]．多くの症例の情報をもとに遺伝子変異の性質と臨床症状について検討され，19q13 領域の広範な欠失は，常に精神発達遅延を合併することが指摘された[4,11]．また，Arg62Trp 変異を持つ患者は重症例が多く，9 例中 8 例が輸血依存性であった．

1999 年に *RPS19* 遺伝子が DBA の原因遺伝子であることが報告されてから，Gazda らによって第 2 の DBA の原因遺伝子が同定されるまでに数年を要したが，その後，次々に RP 遺伝子が DBA の原因遺伝子として同定された．Gazda らは Affymetrix 社の GeneChip Human Mapping 10K Array を用いて，常染色体優性の遺伝形式をもつ DBA の 1 家系のゲノム全体にわたる連鎖解析を行った．その結果，染色体 8q の 17.5 Mb の領域，染色体 10 の 5.8 Mb 及び染色体 6 の 3.8 Mb の領域に DBA と連鎖を認めた[12]．彼らは，特定された領域に存在するリボソームタンパク遺伝子に注目し，*RPS20* と *RPL7*（染色体 8q）と *RPS24*（染色体 10q22–q23）のシークエンス解析を行った．その結果，*RPS20* と *RPL7* は正常であったが，*RPS24* にヘテロ変異を認めた．そこで，210 名の DBA 発端者の解析を行い，さらに 2 名に *RPS24* に遺伝子変異を検出した．*RPS24* 遺伝子変異の頻度は，約 1.4% であった．また，Cmejla らは別のリボソームタンパク遺伝子 *RPS17* の変異を DBA 症例で見出した[13]．点変異（2T>G）のために翻訳開始コードン（ATG）が失われ，一方の *RPS17* の発現が失われる変異であった．

それまでの報告は，全て小サブユニットを構成するリボソームタンパク遺伝子であったが，最近，大サブユニットを構成するリボソームタンパク遺伝子の変異も見出された．Farrar らは，CGH による高感度染色体マッピングとマイクロアレイによる発現解析を駆使して，染色体 3q の欠失をもつ 2 例の DBA 患者の解析から，DBA の原因候補遺伝子として大サブユニットを構成するリボソームタンパク遺伝子 *RPL35A* を同定した．そこで，*RPL35A* の変異が本当に DBA で起こっているかどうかを知るために，148 名の DBA 発端者をスクリーニングし，3 名にヘテロ変異を見出した[14]．さらに，最近，Gazda らは，DBA の症例に別の大サブユニットを構成するリボソームタンパク遺伝子の変異を見出した[15]．彼女らは，これまでに *RPS19*，*RPS24* および *RPL35A* に遺伝子変異のみられない 196 名の DBA 発端者の検体を用いて，遺伝子変異が報告されていない 24 個の RP 遺伝子と 1 例のみの報告があった *RPS17* 遺伝子の解析を行った．その結果，*RPL5* と *RPL11* にヘテロ変異を見出した．*RPL5* と *RPL11* の変異の頻度は，それぞれ 6.6% と 4.8% であった．また，その他にも *RPS7*，*RPS17*，*RPL36*，*RPS15*，*RPS27A* に変異をみとめたが，その頻度はいずれも 1% 未満であった．チェコの DBA registry には，31 名（28 家族）の DBA 症例が登録されているが，この均一な population における *RPL5* および *RPL11* の遺伝子変異の頻度は，それぞれ 21.4% と 7,1% であった[16]．興味あることに，*RPS19* 変異とは対照的に，*RPL5* 変異のある症例には口唇・口蓋裂，先天性心疾患や母指の異常などの多発奇形が，*RPL11* 変異では単独の母指異常が高頻度に認められた．

これまで発見された DBA の遺伝子変異は，すべてリボソームタンパク遺伝子のヘテロ変異であった．これは，DBA の原因がリボソームタンパクの haploinsufficiency によって生じるリボソームの機能不全であることを強く示唆している．

III 本邦における DBA の研究

1. 疫学調査

これまでの DBA の原因遺伝子の研究は，全て海外で行われたものであり，本邦での大規模な解析の報告はなく，この分野の研究は大きく遅れていた．このような背景の中で，平成 21 年から厚労省難治性疾患克服研究事業「DBA の効果的診断法の確立に関する研究班」が立ち上がり，疫学調査，遺伝子解析，バイオマーカーの発見，診断基準の作成を目的に研究を進めた．

平成 21 年度，全国の小児科専門医研修施設（520 施設）および小児血液学会評議員（150 名）を対象に，2000 年 1

Table 1　Summary of sequence changes in 8 RP genes in DBA probands

	American et al	Czech	Italia	Japan
No of probands	272	28	128	76
RPS19	25%	21%	28%	10.5%
RPL5	6.6%	21%	9.3%	7.9%
RPS10	6.4%	ND	ND	1.3%
RPL11	4.8%	7%	9.3%	5.3%
RPS35A	3.5%	ND	0	0%
RPS26	2.6%	ND	ND	1.3%
RPS24	2%	ND	1.6%	0%
RPS17	1%	3.6%	ND	1.3%
Total	52.9%	52.6%	48.2%	27.6%

Table 2　Characteristics of Japanese DBA patients with mutations in *RPS19*

Patient	Malformation status	Response at first steroid therapy	Present therapy
1 proband	growth retardation	response	CR
1 daughter	None	response	CR
25 proband	thmub anomaly, growth retardation etc.	ND	ND
28 proband	thmub anomaly, CHD etc.	response	Steroid dependent
30 proband	thumb anomaly, growth retardation	response	Steroid dependent
30 father	growth retardation	NA	CR
43 proband	thumb anomaly	response	Steroid dependet
44 proband	SFD	response	CR
59 proband	None	response	Steroid dependet
70 proband	thumb anomaly	ND	Transfusion dependent

ND; not done, NA; not available, CR; complete remission

月以降に把握された症例について一次疫学調査を行った．その結果，132例のDBA症例の報告があった．平成23年度には，弘前大学と九州大学の倫理委員会の承認が得られ，二次調査を開始し，現在までに67例の二次調査票の回収があった．

また，平成21年度より，中央診断を伴う日本小児血液学会のDBA登録システムを確立し，登録を開始した．オンラインによる登録が可能であるが，オンライン登録ができない場合は，FAXによる登録も受け付けた．中央診断は，末梢血や骨髄塗抹標本を用いて名古屋大学と聖路加国際病院で行い，遺伝子解析は弘前大学，国立感染研究所で行った．レビュー開始から31ヶ月間で500例がレビューされた．レビュー結果はAAが246例，MDSが53例（先天性骨髄不全症候群（CBFS）4例を含む），JMMLが45例，CBFSが45例，急性白血病が23例，その他137例であった．CBFS 45例の中にDBAが11例含まれていた．DBAと診断された症例については弘前大学小児科に遺伝子解析が依頼され，4例で*RP*遺伝子の変異が確認された．

2. 既知の原因遺伝子の解析

DBAで遺伝子変異が報告されている8遺伝子（*RPS19*, *RPS24*, *RPS17*, *RPS10*, *PS26*, *RPL5*, *RPL11*, *RPL35a*）と5q-症候群の原因遺伝子として最近同定された*RPS14*を解析した[17]．最初に，High resolution melt analysis（HRM）法で遺伝子変異の有無をスクリーニングし，変異陽性と判定された検体をダイレクト・シークエンス法で解析した．これまでに，76家系（83例）を解析し，結果，*RPS19*遺伝子変異が10例（8家系）で検出され，そのうちの2つは新しい遺伝子変異であった．*RPL5*変異は6例，*RPL11*変異は4例，*RPS17*，*RPS10*および*RPS26*変異はそれぞれ1例で検出され，全て新規の変異であった．*RPS19*，*RPL5*と*RPL11*の遺伝子変異の頻度は，それぞれ10.5%，7.9%および5.3%であった（Table 1）．

以上，本邦におけるDBAの発端者76例中21例（27.6%）にRP遺伝子の変異を認めた．

3. 既知の遺伝子変異と表現型

*RPS19*変異をもつ10例中8例に身体異常が認められた（Table 2）．そのうちの5例が拇指の異常を伴っていた．一方，欧米からの報告では*RPS19*変異に奇形を合併する率は40%程度で，拇指の奇形が多いとの報告も無かった．*RPL5*変異の症例も，6例中5例に奇形を認め，さらに，そのう

Table 3 Characteristics of Japanese DBA patients with mutations in *RPL5*

Patient	Malformation status	Response at first steroid therapy	Present therapy
10 proband	cleft palate, thumb anomaly, MR, rib defect, micrognathia, CHD	poor	Transfusion
41 proband	cleft palate, craniofacial abnormalities, CHD (GA, DORV, CoA, LSVC)	ND	Transfusion dependent
55 proband	thmub anomaly, polydactyly	response	Steroid dependet
65 proband	growth retardation	response	Steroid
73 proband	SFD	response	Steroid dependet
74 proband	None	None	Transfusion dependent

ちの2例は重症多発奇形を合併していた（Table 3）．注目すべきことに，口蓋裂を2例に認めた．欧米からの報告でも，*RPL5* 変異の症例は多発奇形の合併が多く，特に口蓋裂，口唇裂を合併することが多いことが示されていたが，今回の解析はそれを裏付ける結果であった[15,16]．

ステロイドは，DBA の治療の根幹であるが，評価可能な症例61例のうち，ステロイドに対する初回反応は73%に見られた．しかし，遺伝子変異による差は認められなかった．

4. DBA の遺伝子コピー数解析法による RP 遺伝子の片アレルの欠失

欧米に比して本邦における RP 遺伝子の変異頻度は低く，約70%の症例では原因遺伝子が不明であることが明らかになった．しかし，片アレルの大きな欠失がある場合，通常のシークエンス解析では，残った正常のアレルの塩基配列のみを読んでしまうため遺伝子異常を検出できない．そこで，Kuramitsu らは，独自に簡便かつ迅速な DBA の遺伝子コピー数解析法（DBA 同期的 q-PCR 法）を開発し，片アレルの欠失の同定を試みた[18]．この方法では，まず，遺伝子上の複数箇所に同一条件で増幅反応ができる PCR プライマーを設定する．もし，大きな欠失が存在し N の状態になっていると，正常の 2N に比べ DNA 量が半分であるため，2N と同じ増幅を得るのに PCR の反応回数が1回多く必要になることを利用して，片アレルの欠失を同定する（Figure 1）．例えば，症例3では，*RPL5* のみが正常に比べ，1サイクルずれていることから，*PRL5* の片アレルの欠失が推定される．DBA で高頻度に変異報告のある遺伝子として，*RPL5*, *L11*, *L35A*, *S7*, *S10*, *S17*, *S19*, *S24*, *S26* の9遺伝子，また，DBA の原因としての重要度は未だ不明であるが論文や学会等において変異報告のあった遺伝子，*RPL9*, *L19*, *L26*, *L28*, *L36*, *S15*, *S27A* の7遺伝子につい

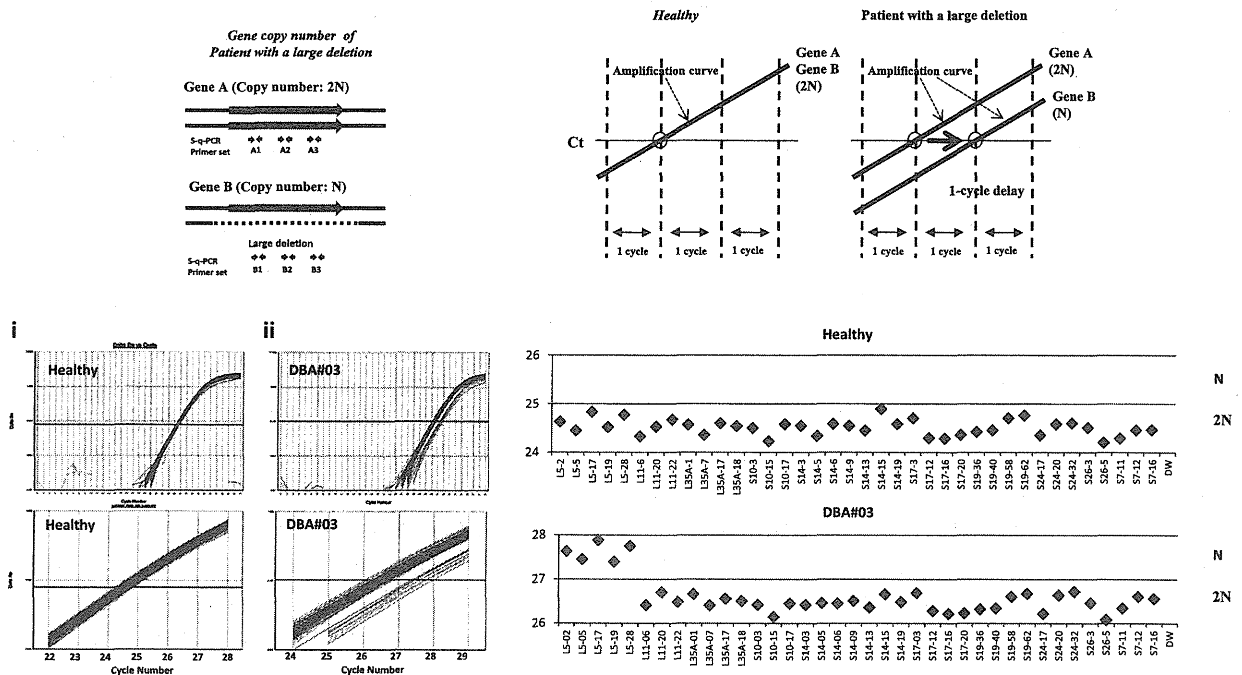

Figure 1 DBA Genomic Copy Number Assay

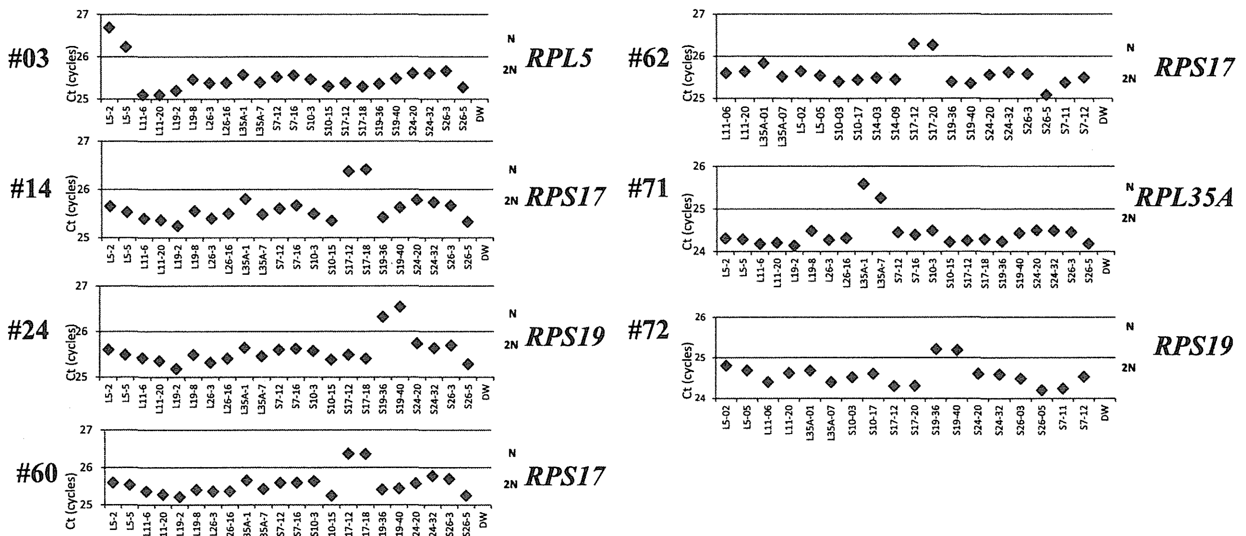

Figure 2　DBA Genomic Copy Number Assay

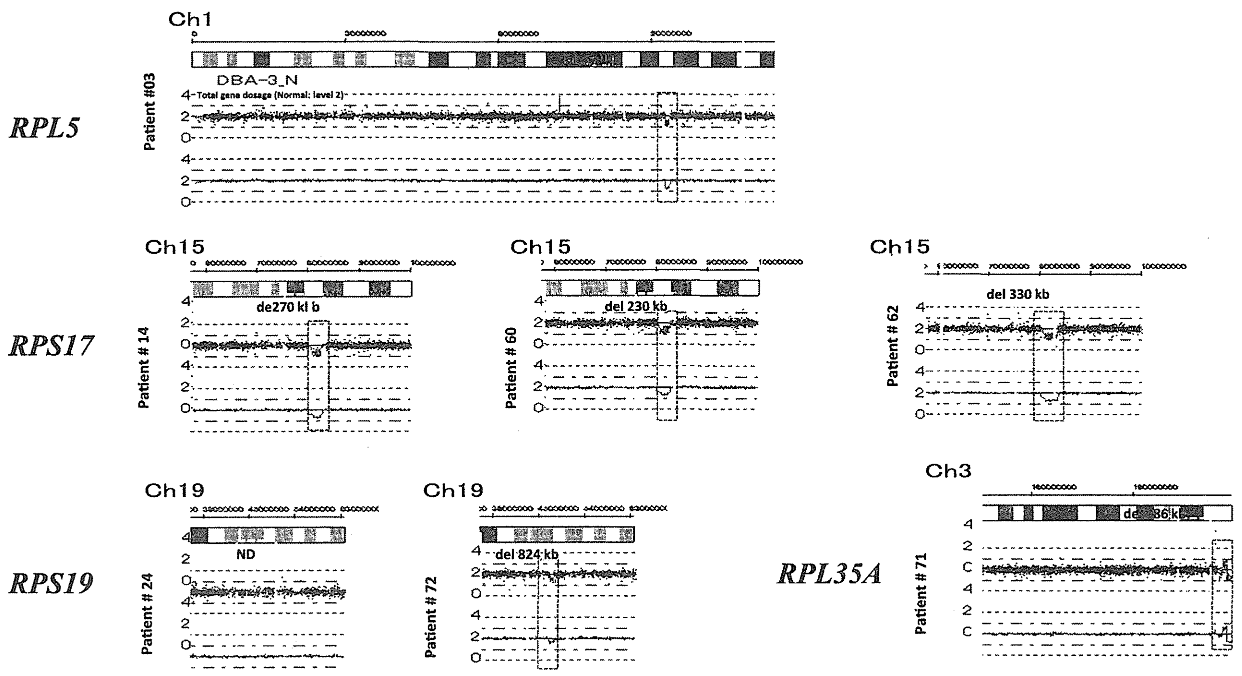

Figure 3　SNP Array

て，遺伝子コピー数測定用のq-PCRプライマーを設定した．弘前大学においてシーケンス解析の結果，変異が検出されなかった検体について，上記の遺伝子の遺伝子コピー数を測定した．その結果，27例中7例で特定の1遺伝子に対するプライマーセット全てが他の遺伝子の増殖曲線のCt値から1サイクルの遅れを示した（Figure 2）．つまり，これら1サイクル遅れた遺伝子は，その他の遺伝子（2N）のコピー数の半分（N）であることを示しており，片アレルの欠失が示された．7例の内訳は，*RPS17*欠失（3例），*RPS19*欠失（2例），*RPL5*欠失（1例），*RPL35A*欠失（1例）であった．

5. SNPアレイによる片アレル欠失の解析

上記の結果を確認するために，SNPアレイ（Affymetrx Gene Chip）とCNAGプログラムで遺伝子コピー数を測定した．その結果，27例中6例でRP遺伝子の片アレル欠損が確認された（Figure 3，Table 4）．これらは，q-PCRの結果と一致していたが，q-PCR法で検出された*RPS19*遺伝子

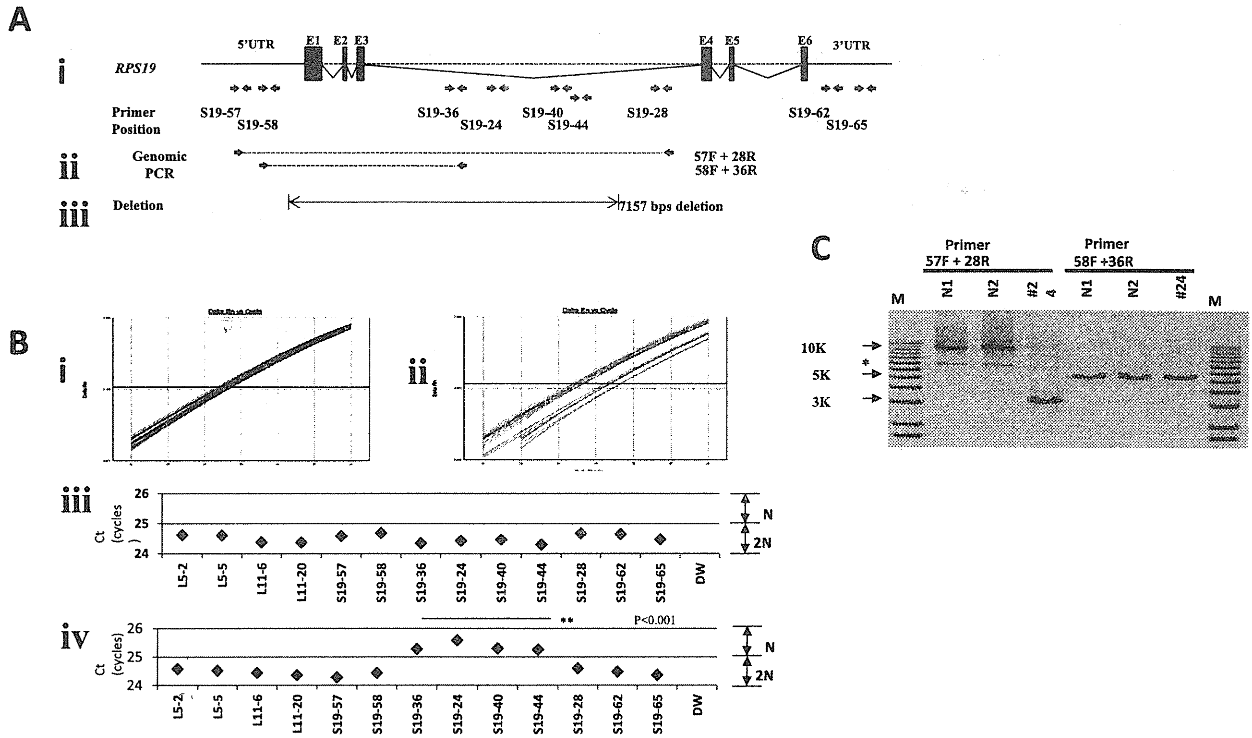

Figure 4　Large deletion in the *RPS19* gene

の大欠失（#24）は検出されなかった（Figure 3）．そこで，*RPS19* に対する q-PCR プライマーを増やし，*RPS19* の遺伝子コピー数を詳細に調べた（Figure 4A）．その結果，*RPS19* の 5'UTR 領域（S19-57, -58）および intron 3 内部から 3'UTR 領域に設定したプライマー（S19-28, -62, -65）ではコピー数が正常値を示したのに対して，intron 3 の内部より上流に設定されたプライマー（S19-36,-24, -40, -44）では片アレルの欠失を示した（Figure 4B）．このことから，*RPS19* 遺伝子の片方のアレルの intron 3 領域付近で小規模な欠失があることが考えられた．そこで，*RPS19* の 5'UTR と intron 3 にプライマーを設定し genomic PCR を行い，欠失領域の同定を試みた．その結果，#24 検体では約 7k 塩基対の欠失が想定されるバンドが電気泳動上で認められた（Figure 4C）．シーケンス解析の結果，5'UTR と intron 3 にある 23 塩基対の相同配列（CGGTGGCTCACACCTGTAATCCCAGCA, nt: −1400 から −1374 および nt: +5758 から +5784）の間で分子内相同組換えが生じ，結果として 7157 塩基の欠失が同定された（Figure 4A）．欠失した領域には，プロモーターおよび exon 1, 2, 3 が含まれることから，このアレルは正常な RPS19 タンパク質をコードしないと考えられた．また，SNP アレイの結果が，q-PCR の結果と一致しなかったのは，SNP プローブが欠失領域に存在しないためであった．

以上の結果より，s-qPCR を用いた片アレル欠失の解析法は，優れた方法であることが示された．

6. 大欠失と表現型

我々が同定した 7 例の DBA の原因遺伝子の片アレル欠失は，その多くがこれまでに報告のないものであった（*RPL5*（1 例），*RPS17*（3 例））．また，今回の解析では，*RPS17* の遺伝子異常が 3 例発見され，これまで欧米では極めて稀とされていたが，興味深いことに日本ではむしろ高頻度で *RPS17* に変異があることが明らかになった．片アレル欠失を有する症例は，共通の表現型を有し，7 例全例が，子宮内発育遅延や低身長などの発育遅延を有していた（Table 5）．欠失を示した 6 検体（#3, #24, #60, #62, #71, #72）について，両親の欠失を同様に DBA 同期 q-PCR 法で調べた．その結果，全ての両親で DBA 原因遺伝子の欠失は認められなかった．つまり，片アレルの欠失の場合は家族性ではなく，孤発性の変異タイプである可能性が示唆された．

7. 未知の遺伝子の検索

上記の解析を行っても原因遺伝子が不明な臨床検体について，次世代シークエンサーを用いた全エクソン解析を行い，新規原因遺伝子の同定を開始した．既知の SNP を除去した後，家族内罹患者や陰性コントロール（非罹患同胞や両親）の全エクソンデータと比較検討することにより，責任変異の候補となる遺伝子を絞り込み，得られた候補遺伝子の変異は，サンガーシークエンス法を用いて確認を行

Table 4 Genes that were found to be deleted using SNP arrays

Patient No.	Large deletion (Q-PCR)	Large deletion (SNP array)	Deletion length	Mutation	Genes deleted
#3	*RPL5*	*RPL5*	858 kb	whole allele loss	*EVI5, RPL5, FAM69A, MTF2, TMED5, CCDC18, DR1*
#14	*RPS17*	*RPS17*	270 kb	whole allele loss	*RPS17, CPEB1, AP3B2, FSD2, WHMM, HOMER2, FAM103A1, C15orf40, BTBD1, TM65F1, HDGFR3, BNC1, SH3GL3, ADAMTSL3, ZSCAN2, SAND2, WDR73, NMB*
#24	*RPS19*	*ND*	7157 bp	Ex 1-3 deletion	*RPS19*
#60	*RPS17*	*RPS17*	230 kb	whole allele loss	*RPS17, CPEB1, AP3B2, FSD2, WHMM, HOMER2, FAM103A1, C15orf40, BTBD1, TM65F1, HDGFR3, BNC1, SH3GL3, ADAMTSL3, ZSCAN2, SAND2, WDR73, NMB*
#62	*RPS17*	*RPS17*	330 kb	whole allele loss	*RPS17, CPEB1, AP3B2, FSD2, WHMM, HOMER2, FAM103A1, C15orf40, BTBD1, TM65F1, HDGFR3, BNC1, SH3GL3,ADAMTSL3, ZSCAN2, SAND2, WDR73, NMB, SEC11A, ZNF592, ALPK3, SLC28A1, PDE8A*
#71	*RPL35A*	*RPL35A*	786 kb	whole allele loss	*BDH, FYTTD1, LRCH3, IQCG, RPL35A, LMLN, FAM157A*
#72	*RPS19*	*RPS19*	824 kb	whole allele loss	*CEACAM5, CEACAM6, CEACAM3, LYPD4, DMRTC2, RPS19, CD79A, ARHGFF1, RABAC1, ATP1A3, GRIK5, ZNF574, POU2F2, DEDD2, ZNF526, GSK3A, ERF, CIC, PAFAH1B3, PRR19, TMEM145, MEGF8, CNFN, LIPE*

Table 5 Characteristics of DBA patients with large deletions

	Patients (Gender)	Inheritance	Age at diagnosis	Physical anamalies
RPS19	24 proband (F)	Sporadic	1 M	Short stature, SGA
	72 proband (M)	Sporadic	0 Y	Thumb anomaly, Flat thenar, testicular hypoplasia, Fetal dydrops
RPS17	14 proband (M)	sporadic	5 Y	Short stature, white spots
	60 proband (F)	sporadic	2 M	SGA
	62 proband (F)	sporadic	1 M	Small ASD, SGA, short stature
RPL5	3 proband (M)	Sporadic	0 Y	Short stature, thumb anomalies
RPL35a	71 proband (M)	Sporadic	0 Y	Thumb anomalies, synostosis of radius and ulna, Cohelia Lange-like face, cleft plate, underdescended testis, short stature, cerebellar hypoplasia, fetal dydrops

う．これまで報告されているDBAの原因遺伝子はすべてリボソーム蛋白遺伝子であったが，全エクソン解析を行った検体の中には，約80種類存在するリボソームタンパク遺伝子に変異のないものが多数みられた．この結果から，リボソームタンパク遺伝子以外のDBA原因遺伝子が存在することが強く示唆された．効率的に原因遺伝子を同定するために，まず，家族内に複数の罹患者がある症例を中心に検索を進める予定である．

8. バイオマーカーの発見

DBAの確定診断にはリボソームタンパク遺伝子変異の同定が有用だが，原因遺伝子が多数あること，遺伝子変異が同定される症例が未だ40～50%であることが大きな問題である．生化学的バイオマーカーとして，赤血球アデノシンデアミナーゼ（eADA）の上昇が知られているが，臨床像，血液学的所見でDBAが強く疑われる症例の10～20%ではeADAが基準値内であり，より高感度にDBAを診断出来る新規バイオマーカーが必要と考えられていた．今回，菅野らは赤血球内の分子XがDBA患者で有意に高値であることを発見し，eADAと併用することで，検討した9家系11例のDBA患者を100%診断し得た（未発表）．このことから，XはDBA診断における新規バイオマーカーとして，極めて有用と考えられた．

IV 結　語

日本のDBA患者で原因遺伝子の片アレル欠失解析を行った結果，DBAでは高頻度（27例中7例）で原因遺伝子の片アレルの欠失が認められた．この結果から，DBAの確定診断のためには，これまで行われてきたシーケンス解析に加えて，コピー数解析を行うことが重要であることが示された．また，遺伝子変異同定率が上昇することによって，日本のDBAの遺伝子型と貧血・奇形等の表現型との相関をより詳しく解析することができるようになると思われる．

DBAに対する治療は副腎皮質ステロイドが第一選択であるが，ステロイド依存や輸血依存症例が全体の約40%存在する．難治例には造血幹細胞移植が行われているが，

本疾患では家族内に発端者と同一の遺伝子異常をもっていても貧血のない軽症例が存在することが知られている．本症は悪性疾患を合併しやすいことから，造血幹細胞移植のドナーを選択する上で軽症例の診断は重要課題になっている．しかし，軽症例も含めたDBA の診断基準の作成は海外でも検討が始まったばかりであり，我が国でも利用できるような診断基準は存在しない．今回のバイオマーカーの発見は，骨髄移植ドナーの選定をする上で非常に重要な情報を与えてくれると思われる．

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ORIGINAL ARTICLE

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

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

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

Keywords Congenital sideroblastic anemia · Myelodysplastic syndrome · ALAS2

Introduction

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

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

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

Materials and methods

Data acquisition

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

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

Diagnostic procedure

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

Genetic analysis of patients with sideroblastic anemia

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

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

Analysis of enzymatic activity of recombinant ALAS2 protein

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

Statistical analysis

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

Results

Epidemiology of sideroblastic anemia

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

Analysis of the pathology of congenital sideroblastic anemia

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

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

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

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

Chromosomal abnormalities of MDS

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

Treatment and outcome

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

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

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

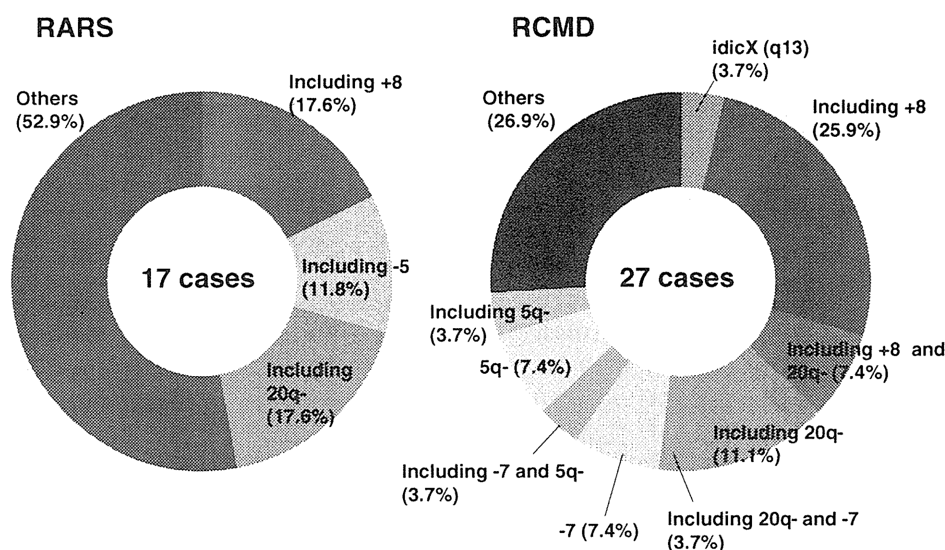

Fig. 1 Chromosomal abnormalities in RARS and RCMD. Data of chromosomal analysis in RARS and RCMD are shown. +8 was most common both in RARS and RCMD. -7 was only seen in RCMD

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

Gene analysis of congenital sideroblastic anemia

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

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

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

Table 3 Congenital sideroblastic anemia (XLSA)

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

[a] % of WT

[b] Present study

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

Discussion

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

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

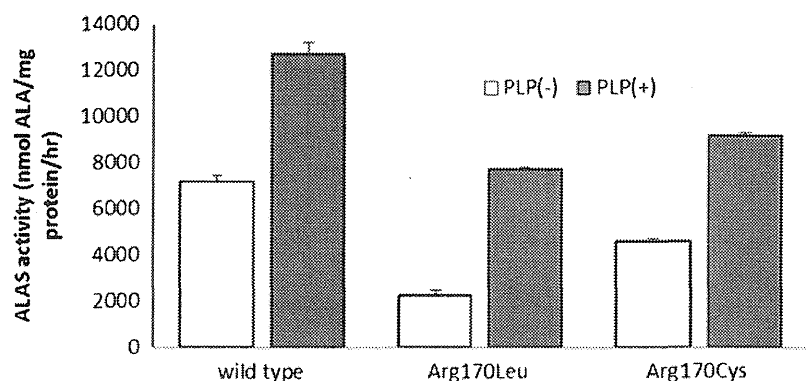

Fig. 2 Enzymatic activity of mutant ALAS2 proteins. Enzymatic activity of wild-type and mutant ALAS2 proteins was measured as described in Materials and Methods. Both of R170L and R170C ALAS2 mutant proteins showed decreased enzymatic activity; however, the activity was partially restored by the addition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

Table 4 Congenital sideroblastic anemia (other than XLSA)

Case number	Age at diag (y.o.)	Gender	Family history	Gene mutation							Hb (g/dl)	MCV (fl)	Response to Vit.B6
				ALAS2	*SLC25A38*	*GLRX5*	*ABCB7*	*SLC19A2*	*PUS1*	*SF3B1*			
11	19	M	–	–	–	–	–	–	–	–	7.8	73.9	–
12	4	M	–	–	–	–	–	–	–	–	6.6	73.6	–
13	0	M	+	–	–	–	–	–	–	–	3.9	65.0	–
14	20	M	+	–	–	–	–	–	–	–	7.6	82.0	+
15	0	M	–	N/D	N/D	N/D	N/D	N/D	N/D	N/D	6.8	88.1	N/D[a]
16	32	M	–	N/D	N/D	N/D	N/D	N/D	N/D	N/D	11.2	69	+
17	36	M	–	N/D	N/D	N/D	N/D	N/D	N/D	N/D	10.8	67.3	+
18	18	F	+	N/D	N/D	N/D	N/D	N/D	N/D	N/D	9.3	96.2	+

N/D not done

[a] Vit.B6 was not administered due to PMPS

+8 appeared to be more common in RCMD. In addition, −7 was identified in four patients with RCMD (14.8 %), whereas it was not identified in RARS. This difference may be related to the poor prognosis of RCMD.

Regarding the responsiveness to pyridoxine treatment among XLSA, 6 of 10 cases responded to Vit.B6 treatment in this study, although the magnitude of response varied among individuals. Thus, as the benefit of treatment of Vit.B6 for XLSA is obvious, a precise diagnosis of XLSA is important. As late-onset XLSA cases have been reported and two patients over 60 years old were found in this study, genetic analysis in sideroblastic anemia patients with microcytic anemia is essential regardless of age.

Focusing on *ALAS2* mutation in XLSA, two patients with the same mutation (c.509G>T), which results in R170L, showed distinct responses to Vit.B6. Edgar et al. [22] reported a Vit.B6 responsive pedigree with XLSA carrying the p.R170L mutation of ALAS2 gene. Furthermore, the crystal structure analysis of ALAS from *Rhodobacter capsulatus* [23] suggests that a missense mutation at Arg170 destabilizes PLP binding, which might be partially restored with excess amounts of PLP. Together with the findings of biochemical analysis in this study, it is strongly suggested that R170L mutation causes pyridoxine-responsive XLSA. However, in consistent with the data of in vitro analysis and clinical course of other R170L patients, case 10 was unresponsive to Vit.B6 treatment. Thus, onset and severity of the disease may be defined by not only the type of mutation but also the environmental and physiological status of the patients. This speculation may be supported by the results that there is a discrepancy between in vitro and in vivo response to Vit.B6 in some cases (Table 3).

The high incidence of XLSA among CSA in the present study was consistent with a previous report in the USA. Bergmann et al. [24] reported genetic analysis of CSA in the USA. In this study, mutations of *ALAS2*, *SLC25A38*, mitochondria DNA, and *PUS1*, were identified in 37, 15, 2.5, and 2.5 % of CSA cases, respectively. The most significant difference from our study was that mutations of the *SLC25A38* gene were frequently found in the USA. Since *SLC25A38* is thought to be a transporter of glycine, which is a substrate for ALAS2 in the first step of heme synthesis, the

Table 5 Mutation of *SF3B1* gene in MDS-RS

Case number	Diagnosis	Age at diagnosis (y.o.)	Gender	Chromosome anomaly	position of *SF3B1* mutation
1	RARS	82	M	–	E622D
2	RARS	57	M	–	N626S
3	RARS	60	M	Complex karyotype, including +8	K700E
4	RARS	60	M	–	K700E
5	RARS	73	F	–	No mutation
6	RARS	74	F	–	H662Q
7	RARS	76	M	–	K700E
8	RARS	67	F	–	K700E
9	RARS	66	M	–	K666E
10	RCMD	50	F	–	No mutation

(–) normal karyotype

pathology of CSA due to mutation of this gene is similar to that of XLSA. Therefore, CSA patients with microcytic anemia, in whom mutations of *ALAS2* gene were not identified, were expected to harbor *SLC25A38* mutation; however, it was not detectable in this study. To date, it has not been reported in Asia, although mutation of the *SLC25A38* gene has been widely reported in the USA, Canada, and Europe. Together with the results of the present study, it is suggested that the causative genes of CSA differ among races and regions.

Recently, mutations of genes involved in splicing machinery were reported in MDS [6]. Among them, *SF3B1*, which is a component of the U2-small nuclear ribonucleoprotein (U2-snRNP) complex [25], was found to be highly mutated in MDS with ring sideroblasts [6]. In this study, *SF3B1* mutation was examined in nine cases of CSA; however, its mutation was not detectable in CSA. These findings suggest that the mechanism for sideroblasts formation may be different between CSA and MDS.

In conclusion, our data showed that XLSA is the most frequent type of CSA; however, onset and severity of the disease may be affected by the environmental and physiological status of the patients. The data, including clinical and genetic analysis, further suggest that genetic background is different between CSA and MDS.

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