◎Fujiwara T, Harigae H.	Pathophysiology and genetic mutations in congenital sideroblastic anemia.	Pediatrics International	55	675-679	2013
Inoue A, Fujiwara T, Okitsu Y, Katsuoka Y, Fukuhara N, Onishi Y, Ishizawa K, Harigae H.	Elucidation of the role of LMO2 in human erythroid cells.	Experimental Hematology	41	1062-1076	2013
Fujiwara T, Okitsu Y, Katsuoka Y, Fukuhara N, Onishi Y, Ishizawa K, Harigae H.	Expression profiling of ETO2-regulated miRNAs in erythroid cells: possible influence on miRNA abundance.	FEBS Open Bio.	3	428-432	2013
◎Hira A, Yabe H, Yoshida K, Okuno Y, Shiraishi Y, Chiba K, Tanaka H, Miyano S, Nakamura J, Kojima S, Ogawa S, Matsuo K, Takata M and Yabe M.	Variant ALDH2 is associated with accelerated progression of bone marrow failure in Japanese Fanconi anemia patients.	Blood	122	3206-3209	3013
○矢部みはる	遺伝性骨髄不全症候群における遺伝子解析と倫理的諸問題	日本小児血液・がん学会雑誌	50	418-420	2013
Horibe K, Saito AM, Takimoto T, Tsuchida M, Manabe A, Shima M, Ohara A, Mizutani S.	Incidence and survival tates of hematological malignancies in Japanese children and adolescents (2006-2010): based on registry data from the Japanese Society of Pediatric Hematology.	Int J Hematol.	98	74-88	2013
Yoshimi A, Kamachi Y, Imai K, Watanabe N, Nakadate H, Kanazawa T, Ozono S, Kobayashi R, Yoshida M, Kobayashi C, Hama A, Muramatsu H, Sasahara Y, Jakob M, Morio T, Ehl S, Manabe A, Niemeyer C, Kojima S.	Wiskott-Aldrich syndrome presenting with a clinical picture mimicking juvenile myelomonocytic leukemia	Pediatr Blood Cancer.	60	836-841	2013
Kato M, Yasui N, Seki M, Kishimoto H, Sato-Otsubo A, Hasegawa D, Kiyokawa N, Hanada R, Ogawa S, Manabe A, Takita J, Koh K.	Aggressive transformation of juvenile myelomonocytic leukemia associated with duplication of oncogenic KRAS due to acquired uniparental disomy.	J Pediatr.	162	1285-1288	2013

◎Fujino H, Doisaki S, Park YD, Hama A, Muramatsu H, Kojima S, Sumimoto S.	Congenital dyserythropoietic anemia type 1 with a novel mutation in the CDAN1 gene previously diagnosed as congenital hemolytic anemia.	Int J Hematol.	97(5)	650-653	2013
○Sakaguchi H, Nakanishi K, Kojima S.	Inherited bone marrow failure syndromes in 2012.	Int J Hematol.	97(1)	20-29	2013
○Viprakasit V, Ekwattanakit S, Riolueang S, Chalaow N, Fisher C, Lower K, Kanno H, Tachavanich K, Bejrachandra S, Saipin J, Juntharaniyom M, Sanpakit K, Tanphaichitr VS, Songdej D, Babbs C, Gibbons RJ, Philipsen S, Higgs DR.	Mutations in Kruppel-like factor 1 cause transfusion-dependent hemolytic anemia and persistence of embryonic globin gene expression.	Blood			2014 [Epub ahead of print]
Tsuzuki S, Akahira-Azuma M, Kaneshige M, Shoya K, Hosokawa S, Kanno H, Matsushita T.	A Japanese neonatal case of glucose-6-phosphate dehydrogenase deficiency presenting as severe jaundice and hemolytic anemia without apparent trigger.	Springerplus	2	434	2013
Tomida J, Itaya A, Shigechi T, Unno J, Uchida E, Ikura M, Masuda Y, Matsuda S, Adachi J, Kobayashi M, Meetei AR, Maehara Y, Yamamoto KI, Kamiya K, Matsuura A, Matsuda T, Ikura T, Ishiai M, Takata M.	A novel interplay between the Fanconi anemia core complex and ATR-ATRIP kinase during DNA cross-link repair.	Nucleic Acids Res.	41(14)	6930-6941	2013
Hosono Y, Abe T, Ishiai M, Islam MN, Arakawa H, Wang W, Takeda S, Ishii Y, Takata M, Seki M, Enomoto T.	Tumor suppressor RecQL5 controls recombination induced by DNA crosslinking agents.	Biochim Biophys Acta.			2014 in press
Inoue H, Ohga S, Kusuda T, Kitajima J, Kinjo T, Ochiai M, Takahata Y, Honjo S, Hra T.	Serum neutrophil gelatinase-associated lipocalin as a predictor of the development of bronchopulmonary dysplasia in preterm infants.	Early Hum Dev.	89(6)	425-419	2013

Aizawa Y, Kanno H, Kawamichi Y, Matsuda Y, Ohta H, Fujii H, Matsui H, Saito K.	Enhanced expression of myogenic differentiation factors and skeletal muscle proteins in human amnion-derived cells via the forced expression of MYOD1.	Brain Dev.	35	349-355	2013
Ishida Y, Maeda M, Urayama KY, Kiyotani C, Aoki Y, Kato Y, Goto S, Sakaguchi S, Sugita K, Tokuyama M, Nakadate N, Ishii E, Tsuchida M, Ohara A.	Secondary cancers among children with acute lymphoblastic leukaemia treated by the Tokyo Children's Cancer Study Group protocols: a retrospective cohort study.	Br J Haematol.			2013
Jeong DC, Chung NG, Cho B, Zou Y, Ruan M, Takahashi Y, Muramatsu H, Ohara A, Kosaka Y, Yang W, Kim HK, Zhu X, Kojima S.	Long-term outcome after immunosuppressive therapy with horse or rabbit antithymocyte globulin and cyclosporine for severe aplastic anemia in children.	Haematologica			2013 in press
Kato M, Koh K, Manabe A, Saito T, Hasegawa D, Isoyama K, Kinoshita A, Maeda M, Okimoto Y, Kajiwara M, Kaneko T, Sugita K, Kikuchi A, Tsuchida M, Ohara A.	No impact of high-dose cytarabine and asparaginase as early intensification with intermediate-risk paediatric acute lymphoblastic leukaemia: results of randomized trial TCCSG study L99-15.	Br J Haematol.			2013
Kiyokawa N, Iijima K, Tomita O, Miharu M, Hasegawa D, Kobayashi K, Okita H, Kajiwara M, Shimada H, Inukai T, Makimoto A, Fukushima T, Nanmoku T, Koh K, Manabe A, Kikuchi A, Sugita K, Fujimoto J, Hayashi Y, Ohara A.	Significance of CD66c expression in childhood acute lymphoblastic leukemia.	Leuk Res.	38	42-48	2013
○Takahashi Y, Muramatsu H, Sakata N, Hyakuna N, Hamamoto K, Kobayashi R, Ito E, Yagasaki H, Ohara A, Kikuchi A, Morimoto A, Yabe H, Kudo K, Watanabe K, Ohga S, Kojima S.	Rabbit antithymocyte globulin and cyclosporine as first-line therapy for children with acquired aplastic anemia.	Blood	121	862-863	2013

矢部普正	小児におけるGVHD制御	血液フロンティア	23	57-64	2013
Yagasaki H, Shichino H, Ohara A, Kobayashi R, Yabe H, Ohga S, Hamamoto K, Ohtsuka Y, shimada H, Inoue M, Muramatsu H, Takahashi Y,l Kojima S.	Immunosuppressive therapy with horse anti-thymocyte globulin and cyclosporine as treatment for fulminant aplastic anemia in children.	Ann Hematol.			2013 [Epub ahead of print]
○YadavV.G, Chakraborty A, Uechi T, Kenmochi N.	Ribosomal protein deficiency causes Tp53-independent erythropoiesis failure in zebrafish.	Int J Biochem Cell Biol.			2014 in press
○剣持直哉	リボソーム病－リボソーム合成の異常と疾患－	生化学	85(10)	909-915	2013

VI. 研究成果の刊行物・別冊

Extensive gene deletions in Japanese patients with Diamond-Blackfan anemia

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Fifty percent of Diamond-Blackfan anemia (DBA) patients possess mutations in genes coding for ribosomal proteins (RPs). To identify new mutations, we investigated large deletions in the RP genes *RPL5*, *RPL11*, *RPL35A*, *RPS7*, *RPS10*, *RPS17*, *RPS19*, *RPS24*, and *RPS26*. We developed an easy method based on quantitative-PCR in which the threshold cycle correlates to gene copy number. Using this approach, we were able to diagnose 7 of 27 Japanese patients (25.9%) possessing mutations that were not detected by sequencing. Among these large deletions, similar results were obtained with 6 of 7 patients screened with a single nucleotide polymorphism array. We found an extensive intragenic deletion in *RPS19*, including exons 1-3. We also found 1 proband with an *RPL5* deletion, 1 patient with an *RPL35A* deletion, 3 with *RPS17* deletions, and 1 with an *RPS19* deletion. In particular, the large deletions in the *RPL5* and *RPS17* alleles are novel. All patients with a large deletion had a growth retardation phenotype. Our data suggest that large deletions in RP genes comprise a sizable fraction of DBA patients in Japan. In addition, our novel approach may become a useful tool for screening gene copy numbers of known DBA genes. (*Blood*. 2012;119(10): 2376-2384)

Introduction

Diamond-Blackfan anemia (DBA; MIN# 105650) is a rare congenital anemia that belongs to the inherited BM failure syndromes, generally presenting in the first year of life. Patients typically present with a decreased number of erythroid progenitors in their BM.[1] A main feature of the disease is red cell aplasia, but approximately half of patients show growth retardation and congenital malformations in the craniofacial, upper limb, cardiac, and urinary systems. Predisposition to cancer, in particular acute myeloid leukemia and osteogenic sarcoma, is also characteristic of the disease.[2]

Mutations in the *RPS19* gene were first reported in 25% of DBA patients by Draptchinskaia et al in 1999.[3] Since that initial finding, many genes that encode large (RPL) or small (RPS) ribosomal subunit proteins were found to be mutated in DBA patients, including *RPL5* (approximately 21%), *RPL11* (approximately 9.3%), *RPL35A* (3.5%), *RPS7* (1%), *RPS10* (6.4%), *RPS17* (1%), *RPS24* (2%), and *RPS26* (2.6%).[4-7] To date, approximately half of the DBA patients analyzed have had a mutation in one of these genes. Konno et al screened 49 Japanese patients and found that 30% (12 of 49) carried mutations.[8] In addition, our data showed that 22 of 68 DBA patients (32.4%) harbored a mutation in ribosomal protein (RP) genes (T.T., K.T., R.W., and E.I., unpublished observation, April 16, 2011). These abnormalities of RP genes cause defects in ribosomal RNA processing, formation of either the large or small ribosome subunit, and decreased levels of polysome formation,[4-6,9-12] which is thought to be one of the mechanisms for impairment of erythroid lineage differentiation.

Although sequence analyses of genes responsible for DBA are well established and have been used to identify new mutations, it is estimated that approximately half of the mutations remain to be determined. Because of the difficulty of investigating whole allele deletions, there have been few reports regarding allelic loss in DBA, and they have only been reported for *RPS19* and *RPL35A*.[3,6,13] However, a certain percentage of DBA patients are thought to have a large deletion in RP genes. Therefore, a detailed analysis of allelic loss mutations should be conducted to determine other RP genes that might be responsible for DBA.

In the present study, we investigated large deletions using our novel approach for gene copy number variation analysis based on quantitative-PCR and a single nucleotide polymorphism (SNP) array. We screened Japanese DBA patients and found 7 patients with a large deletion in an allele in *RPL5*, *RPL35A*, *RPS17*, or *RPS19*. Interestingly, all of these patients with a large deletion had a phenotype of growth retardation, including short stature and

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Table 1. Primers used for synchronized quantitative-PCR (s-q-PCR) of RPL proteins

Gene	Primer name	Sequence	Primer name	Sequence	Size, bp
RPL5	L5-02F	CTCCCAAAGTGCTTGAGATTACAG	L5-02R	CACCTTTTCCTAACAAATTCCCAAT	132
	L5-05F	AGCCCTCCAACCTAGGTGACA	L5-05R	GAATTGGGATGGGCAAGAACT	102
	L5-17F	TGAACCCTTGCCCTAAAACATG	L5-17R	TCTTGGTCAGGCCCTGCTTA	105
	L5-19F	ATTGTGCAAACTCGATCACTAGCT	L5-19R	GTGTCTGAGGCTAACACATTTCCAT	103
	L5-21F	GTGCCACTCTCTTGGACAAACTG	L5-21R	CATAGGGCCAAAAGTCAAATAGAAG	102
	L5-28F	TCCACTTTAGGTAGGCGAAACC	L5-28R	TCAGATTTGGCATGTACCTTTCA	102
RPL11	L11-06F	GCACCCACATGGCTTAAAGG	L11-6R	CAACCAACCCATAGGCCAAA	102
	L11-20F	GAGCCCCCTTTCTCAGATGATA	L11-20R	CATGAACTTGGGCTCTGAATCC	109
	L11-22F	TATGTGCAGATAAGAGGGCAGTCT	L11-22R	ATACAGATAAGGAAACTGAGGCAGATT	98
RPL19	L19-02F	TGGCCTCTCATAAAGGAAATCTCT	L19-02R	GGAATGCAGGCAAGTTACTCTGTT	103
	L19-08F	TTTGAAGGCAAGAAATAAGTTCCA	L19-08R	AGCACATCACAGAGTCCAAATAGG	107
	L19-16F	GGTTAGTTGAAGCAGGAGCCTTT	L19-16R	TGCTAGGGAGACAGAAGCACATC	102
	L19-19F	GGACCAGTAGTTGTGACATCAGTTAAG	L19-19R	CCCATTTGTAACCCCCACTTG	106
RPL26	L26-03F	TCCAAAGAGCTGAGACAGAAGTACA	L26-03R	TCCATCAAGACAACGAGAACAAGT	102
	L26-16F	TTTGAGAATGCTTGAGAGAAGGAA	L26-16R	TTCCAGCACATGTAAAATCAAGGA	102
	L26-18F	ATGTTTTAATAAGCCCTCCAGTTGA	L26-18R	GAGAACAGCAAGTTGAAAGGTTCA	102
	L26-20F	GGGCTTTGCTTGATCACTCTAGA	L26-20R	AGGGAGCCCGAAAACATTTAC	104
RPL35A	L35A-01F	TGTGGCTTCTATTTTGCGTCAT	L35A-01R	GGAATTACCTCCTTTATTGCTTACAAG	121
	L35A-07F	TTTCCGTTCTGTCTATTGCTGTGT	L35A-07R	GAACCCTGAGTGGAGGATGTTC	113
	L35A-17F	GCCCACAACCTCCAGAGAATC	L35A-17R	GGATCACTTGAGGCCAGGAAT	104
	L35A-18F	TTAGGTGGGCTTTTCAGTCTCAA	L35A-18R	ATCTCCTGATTCCCCAACTTTGT	102
RPL36	L36-02F	CCGCTCTACAAGTGAAGAAATTCTG	L36-02R	CTCCCTCTGCCTGTGAAATGA	102
	L36-04F	TGCGTCCTGCCAGTGTTG	L36-04R	GGGTAGCTGTGAGAACCAAGGT	105
	L36-17F	CCCCTTGAAAGGACAGCAGTT	L36-17R	TTGGACACCAGGCACAGACTT	114

Table 2. Primers used for s-q-PCR of RPS proteins

Gene	Primer name	Sequence	Primer name	Sequence	Size, bps
RPS7	S7-11F	GCGCTGCCAGATAGGAAATC	S7-11R	TTAGGGAGCTGCCTTACATATGG	102
	S7-12F	ACTGGCAGTTCTGTGATGCTAAGT	S7-12R	ACTCTTGCTCATCTCCAAAACCA	102
	S7-16F	GTGTCTGTGCCAGAAAGCTTGA	S7-16R	GAACCATGCAAAAGTGCCAATAT	112
RPS10	S10-03F	CTACGGTTTTGTGTGGGTCACTT	S10-03R	CATCTGCAAGAAGGAGACGATTG	102
	S10-15F	GTTGGCCTGGAGTCGTGATTT	S10-15R	ATTCCAAGTGCACCATTTCCTT	101
	S10-17F	AATGGTGTTTAGGCCAACGTTAC	S10-17R	TTTGAACAGTGGTTTTTGTGCAT	100
RPS14	S14-03F	GAATTCCAAACCCTTCTGCAAA	S14-03R	TTGCTTCATTTACTCCTCAAGACATT	104
	S14-05F	ACAACCAGCCCTCTACCTCTTTT	S14-05R	GGAAGACGCCGGCATTATT	102
	S14-06F	CGCCTCTACCTCGCCAAAC	S14-06R	GGGATCGGTGCTATTGTTATTCC	102
	S14-09F	GCCATCATGCCGAAACATACT	S14-09R	AACGCGCCACAGGAGAGA	102
	S14-13F	ATCAGGTGGAGCACAGGAAAAC	S14-13R	GCGAGGGAGCTGCTTGATT	111
	S14-15F	AGAAGTTTTAGTGAGGCAGAAATGAGA	S14-15R	TCCCCTGGCTATTAAATGAAACC	102
	S14-19F	GATGAATTGTCCTTTCCTCCATTC	S14-19R	TAGGCGGAAACCAAAAATGCT	102
RPS15	S15-11F	CTCAGCTAATAAAGGCGCACATG	S15-11R	CCTCACACCACGAACCTGAAG	108
	S15-15F	GGTTGGAGAACATGGTGAGAACTA	S15-15R	CACATCCCTGGGCCACTCT	108
RPS17	S17-03F	ACTGCTGTCGTGGCTCGATT	S17-03R	GATGACCTGTTCTTCTGGCCTTA	121
	S17-05F	GAAAACAGATACAAATGGCATGGT	S17-05R	TGCCTCCCACTTTTCCAGAGT	114
	S17-12F	CTATGTGTAGGAGGTCCCAGGATAG	S17-12R	CCACCTGGTACTGAGCACATGT	102
	S17-16F	TAGCGGAAGTTGTGTGCATTG	S17-16R	CAAGAACAGAAGCAGCCAAGAG	102
	S17-18F	TGGCTGAATCTGCCTGCTT	S17-18R	GCCTTGTATGTACCTGGAAATGG	103
	S17-20F	GGGCCCTTCACAAATGTTGA	S17-20R	GCAAAACTCTGTCCCTTTGAGAA	101
RPS19	S19-24F	CCATCCCAAGAATGCACACA	S19-24R	CGCCGTAGCTGGTACTCATG	120
	S19-28F	GACACACCTGTTGAGTCCTCAGAGT	S19-28R	GCTTCTATTAACTGGAGCACACATCT	114
	S19-36F	CTCTTGAGGGTGGTCTGGAAAT	S19-36R	GTCTTTGCGGGTTCTTCCTCTAC	102
	S19-40F	GGAACGGTGTCAGGATTCAAG	S19-40R	AGCGGCTGTACACCAGAAATG	101
	S19-44F	CTGAGGTTGAGTGTCCCATTTCT	S19-44R	GCACCGGGCCTCTGTTATC	104
	S19-57F	CAGGGACACAGTGCTGAGAAACT	S19-57R	TGAGATGTCCCATTTTCACTATTGTT	101
	S19-58F	CATGATGTTAGCTCCGTTGCATA	S19-58R	ATTTTGGGAAGAGTGAAGCTTAGGT	102
	S19-62F	GCAACAGAGCGAGACTCCATTT	S19-62R	AGCACTTTTCGGCACTTACTTCA	102
	S19-65F	ACATTTCCCAGAGCTGACATGA	S19-65R	TCGGGACACCTAGACCTTGCT	102
RPS24	S24-17F	CGACCACGTCTGGCTTAGAGT	S24-17R	CCTTCATGCCCAACCAAGTC	101
	S24-20F	ACAAGTAAGCATCATCACCTCGAA	S24-20R	TTTCCCTCACAGCTATCGTATGG	105
	S24-32F	GGGAAATGCTGTGTCCACATACT	S24-32R	CTGGTTTCATGGCTCCAGAGA	105
RPS26	S26-03F	CGCAGCAGTCAGGGACATTT	S26-03R	AAGTTGGGCGAAGGCTTTAAG	104
	S26-05F	ATGGAGGCCGTCTAGTTTGGT	S26-05R	TGCCTACCCTGAACCTTGCT	102
RPS27A	S27A-09F	GCTGGAGTGCATTCGCTTGT	S27A-09R	CACGCCTGTAATCCCCACTAA	102
	S27A-12F	CAGGCTTGGTGTGCTGTGACT	S27A-12R	ACGTCCATCTTCCAGCTGCTT	103
	S27A-18F	GGGTTTTTCCTGTTTGGTATTTGA	S27A-18R	AAAGGCCAGCTTTGCAAGTG	111
	S27A-22F	TTACCATATTGCCAGTCTTTCCATT	S27A-22R	TTCATATGCATTTGCACAAACTGT	106

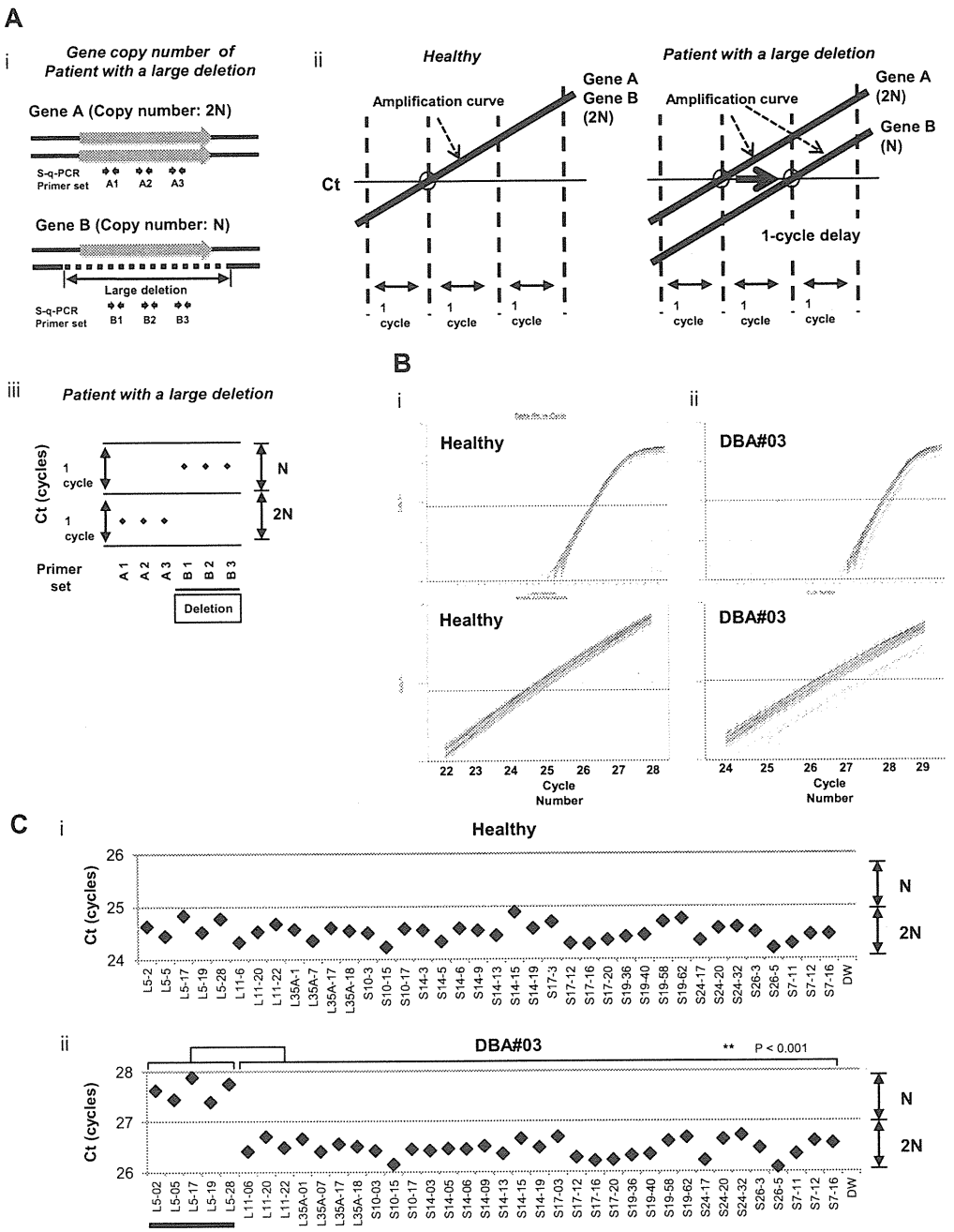

Figure 1. s-q-PCR can determine a large gene deletion in DBA. (A) Concept of the DBA s-q-PCR assay. The difference in gene copy number between a healthy sample and that with a large deletion is 2-fold (i). When all genomic s-q-PCR for genes of interest synchronously amplify DNA fragments, a 2-fold difference in the gene copy number is detected by a 1-cycle difference of the Ct scores of the s-q-PCR amplification curves (ii). Also shown is a dot plot of the Ct scores (iii). (B) Results of the amplification curves of s-q-PCR performed with a healthy person (i) and a DBA patient (patient 3; ii). The top panel shows the results of PCR cycles; the bottom panel is an extended graph of the PCR cycles at logarithmic amplification. (C) Graph showing Ct scores of s-q-PCR. If all specific primer sets for DBA genes show a 1-cycle delay relative to each other, this indicates a large deletion in the gene. Gene primer sets with a large deletion are underlined in the graph. $^{**}P < .001$.

small-for-gestational age (SGA), which suggests that this is a characteristic of DBA patients with a large gene deletion in Japan.

Methods

Patient samples

Genomic DNA was extracted using the GenElute Blood Genomic DNA Kit (Sigma-Aldrich) according to the manufacturer's protocol. Clinical manifestation of patients from a Japanese DBA genomic library are listed elsewhere or are as reported by Konno et al.[8] The study was approved by the institutional review board at the National Institute of Infectious Diseases and Hirosaki University.

DBA gene copy number assay by s-q-PCR

For s-q-PCR, primers were designed using Primer Express Version 3.0 software (Applied Biosystems). Primers are listed in Tables 1 and 2. Genomic DNA in water was denatured at 95°C for 5 minutes and

immediately cooled on ice. The composition of the s-q-PCR mixture was as follows: 5 ng of denatured genomic DNA, 0.4mM forward and reverse primers, 1× SYBR Premix Ex Taq II (Takara), and 1× ROX reference dye II (Takara) in a total volume of 20 μL (all experiments were performed in duplicate). Thermal cycling was performed using the Applied Biosystems 7500 fast real-time PCR system. Briefly, the PCR mixture was denatured at 95°C for 30 seconds, followed by 35 cycles of 95°C for 5 seconds, 60°C for 34 seconds, and then dissociation curve measurement. Threshold cycle (Ct) scores were determined as the average of duplicate samples. The technical errors of Ct scores in the triplicate analysis were within 0.2 cycles (supplemental Figure 1, available on the *Blood* Web site; see the Supplemental Materials link at the top of the online article). The sensitivity and specificity of this method was evaluated with 15 healthy samples. Any false positive was not observed in all primer sets in all healthy samples (supplemental Figure 2). We performed direct sequencing of the s-q-PCR products. The results of the sequence analysis were searched for using BLAST to confirm uniqueness. Sequence data were obtained from GenBank (http://www.ncbi.nlm.nih.gov/gene/) and Ensemble Genome Browser (http://uswest.ensembl.org).

Genomic PCR

Genomic PCR was performed using KOD FX (Toyobo) according to the manufacturer's step-down PCR protocol. Briefly, the PCR mixture contained 20 ng of genomic DNA, 0.4mM forward and reverse primers, 1mM dNTP, 1× KOD FX buffer, and 0.5 U KOD FX in a total volume of 25 μL in duplicate. Primers are given in supplemental Figure 3 and Table 2. PCR mixtures were denatured at 94°C for 2 minutes, followed by 4 cycles of 98°C for 10 seconds, 74°C for 12 minutes, followed by 4 cycles of 98°C for 10 seconds, 72°C for 12 minutes followed by 4 cycles of 98°C for 10 seconds, 70°C for 12 minutes, followed by 23 cycles of 98°C for 10 seconds and 68°C for 12 minutes. PCR products were loaded on 0.8% agarose gels and detected by LAS-3000 (Fujifilm).

DNA sequencing analysis

The genomic PCR product was purified by the GenElute PCR clean-up kit (Sigma-Aldrich) according to the manufacturer's instructions. Direct sequencing was performed using the BigDye Version 3 sequencing kit. Sequences were read and analyzed using a 3120x genetic analyzer (Applied Biosystems).

SNP array-based copy number analysis

SNP array experiments were performed according to the standard protocol of GeneChip Human Mapping 250K Nsp arrays (Affymetrix). Microarray data were analyzed for determination of the allelic-specific copy number using the CNAG program, as described previously.[14] All microarray data are available at the EGA database (www.ebi.ac.uk/ega) under accession number EGAS00000000105.

Results

Construction of a convenient method for RP gene copy number analysis based on s-q-PCR

We focused on the heterozygous large deletions in DBA-responsible gene. The difference in copy number of genes between a mutated DBA allele and the intact allele was 2-fold (N and 2N; Figure 1Ai). If each PCR can synchronously amplify DNA fragments when the template genomic DNA used is of normal karyotype, it is possible to conveniently detect a gene deletion with a 1-cycle delay in s-q-PCR analysis (Figure 1Aii-iii).

Table 3. Summary of mutations and the mutation rate observed in Japanese DBA patients

Gene	Sequencing analysis
RPS19	10
RPL5	6
RPL11	3
RPS17	1
RPS10	1
RPS26	1
RPL35A	0
RPS24	0
RPS14	0
Mutations, n (%)	22 (32.4%)
Total analyzed, N	68

To apply this strategy for allelic analysis of DBA, we prepared primers for 16 target genes, *RPL5*, *RPL11*, *RPL35A*, *RPS10*, *RPS19*, *RPS26*, *RPS7*, *RPS17*, *RPS24*, *RPL9*, *RPL19*, *RPL26*, *RPL36*, *RPS14*, *RPS15*, and *RPS27A*, under conditions in which the Ct of s-q-PCR would occur within 1 cycle of that of the other primer sets (Tables 1 and 2). At the same time, we defined the criteria of a large deletion in our assay as follows. If multiple primer sets for one gene showed a 1-cycle delay from the other gene-specific primer set at the Ct score, we assumed that this represented a large deletion. As shown in Figure 1Bii and 1Cii, the specific primer sets for *RPL5* (L5-02, L5-05, L5-17, L5-19, and L5-28) detected a 1-cycle delay with respect to the mutated allele of patient 3. This assessment could be verified by simply confirming the difference of the cycles with the s-q-PCR amplification curves.

Study of large gene deletions in a Japanese DBA genomic DNA library

Sixty-eight Japanese DBA patients were registered and blood genomic DNA was collected at Hirosaki University. All samples were first screened for mutations in *RPL5*, *L11*, *L35A*, *S10*, *S14*, *S17*, *S19*, and *S26* by sequencing. Among these patients, 32.4% (22 of 68) had specific DBA mutations (Table 3 and data not shown). We then screened for large gene deletions in 27 patients from the remaining 46 patients who did not possess mutations as determined by sequencing (Table 4).

When we performed the s-q-PCR DBA gene copy number assay, 7 of 27 samples displayed a 1-cycle delay of Ct scores: 1 patient had *RPL5* (patient 14), 1 had *RPL35A* (patient 71), 3 had *RPS17* (patients 3, 60, 62), and 2 had *RPS19* (patients 24 and 72; Figure 2 and Table 4). Among these patients, the large deletions in the *RPL5* and *RPS17* genes are the first reported cases of allelic deletions in DBA. From these results, we estimate that a sizable number of Japanese DBA patients have a large deletion.

Based on our findings, the rate of large deletions was approximately 25.9% (7 of 27) in a category of unspecified gene mutations. Such mutations have typically gone undetected by conventional sequence analysis. We could not find any additional gene deletions in the analyzed samples.

Confirmation of the gene copy number for DBA genes by genome-wide SNP array

We performed genome-wide copy number analysis of the 27 DBA patients with a SNP array to confirm our s-q-PCR results. SNP array showed that patient 3 had a large deletion in

Table 4. Characteristics of DBA patients tested

Patient no.	Age at diagnosis	Sex	Hb, g/dL	Large deletion by s-q-PCR	Large deletion by SNP array	Inheritance	Malformations	Response to first steroid therapy
Patients with a large deletion in RP genes								
3*†	1 y	M		RPL5	RPL5	Sporadic	Short stature, thumb anomalies	Response
14*	5 y	M	5.5	RPS17	RPS17	Sporadic	White spots, short stature	Response
24*†	1 mo	F	5.5	RPS19	ND	Sporadic	Short stature, SGA	Response
60*†	2 mo	F	2.4	RPS17	RPS17	Sporadic	SGA	NT
62*†	1 mo	F	6.2	RPS17	RPS17	Sporadic	Small ASD, short stature, SGA	Response
71	0 y	M	5.3	RPL35A	RPL35A	Sporadic	Thumb anomalies, synostosis of radius and ulna, Cohelia Lange-like face, cleft palate, underdescended testis, short stature, cerebellar hypoplasia, fetal hydrops	NT
72†	0 y	M	2	RPS19	RPS19	Sporadic	Thumb anomalies, flat thenar, testicular hypoplasia, fetal hydrops, short stature, learning disability	No
Patients without a large deletion in RP genes								
5*	1 y	F	3.1	ND	ND	Sporadic	ND	Response
15*	1 mo	F	1.6	ND	ND	Sporadic	ND	Response
21*	1 y	F	2.6	ND	ND	Sporadic	ND	Response
26*	1 y 1 mo	F	8	ND	ND	Sporadic	Congenital hip dislocation, spastic quadriplegia, hypertelorism, nystagmus, short stature, learning disability	Response
33*	2 mo	F	1.3	ND	ND	Sporadic	ND	Response
36*	0 y	M	8.2	ND	ND	Familial	ND	Response
37*	4 y	M	6.1	ND	ND	Sporadic	Hypospadias, underdescended testis, SGA	NT
45*	5 d	M	5.1	ND	ND	Sporadic	Short stature, microcephaly, mental retardation, hypogammaglobulinemia	Poor
50*	2 m	F	3.4	ND	ND	Familial	ND	Response
61*	9 m	M	4	ND	ND	Sporadic	ND	Response
63*	0 y	M	6.8	ND	ND	Sporadic	Micrognathia, hypertelorism, short stature	Response
68	1 y 4 mo	M	5.9	ND	ND	Sporadic	ND	NT (CR)
69	1 y	M	9.3	ND	ND	Sporadic	ND	Response
76	0 y	M	4	ND	ND	Sporadic	ND	Response
77	0 y	M	7.8	ND	ND	Familial	Short stature	No
83	9 mo	F	3	ND	ND	Sporadic	ND	NT
90	10 mo	M	9	ND	ND	Sporadic	ND	No
91	0 y	F	3.8	ND	ND	Sporadic	ND	Response
92	2 mo	M	3.7	ND	ND	Sporadic	ASD, PFO, melanosis, underdescended testis, SGA, short stature	Response
93	11 mo	M	2.2	ND	ND	Sporadic	White spots, senile face, corneal opacity, underdescended testis, syndactyly, ectrodactyly, flexion contracture, extension contracture	Response

ND indicates not detected; NT, not tested; CR, complete remission; ASD, atrial septal defect; and PFO, persistent foramen ovale.

*Status data of Japanese probands 3 to 63 is from a report by Konno et al.[6]

†Large deletions of the parents of 5 DBA patients (3, 24, 60, 62, and 72) were analyzed by s-q-PCR, but there were no deletions in DBA genes in any of the 5 pairs of parents.

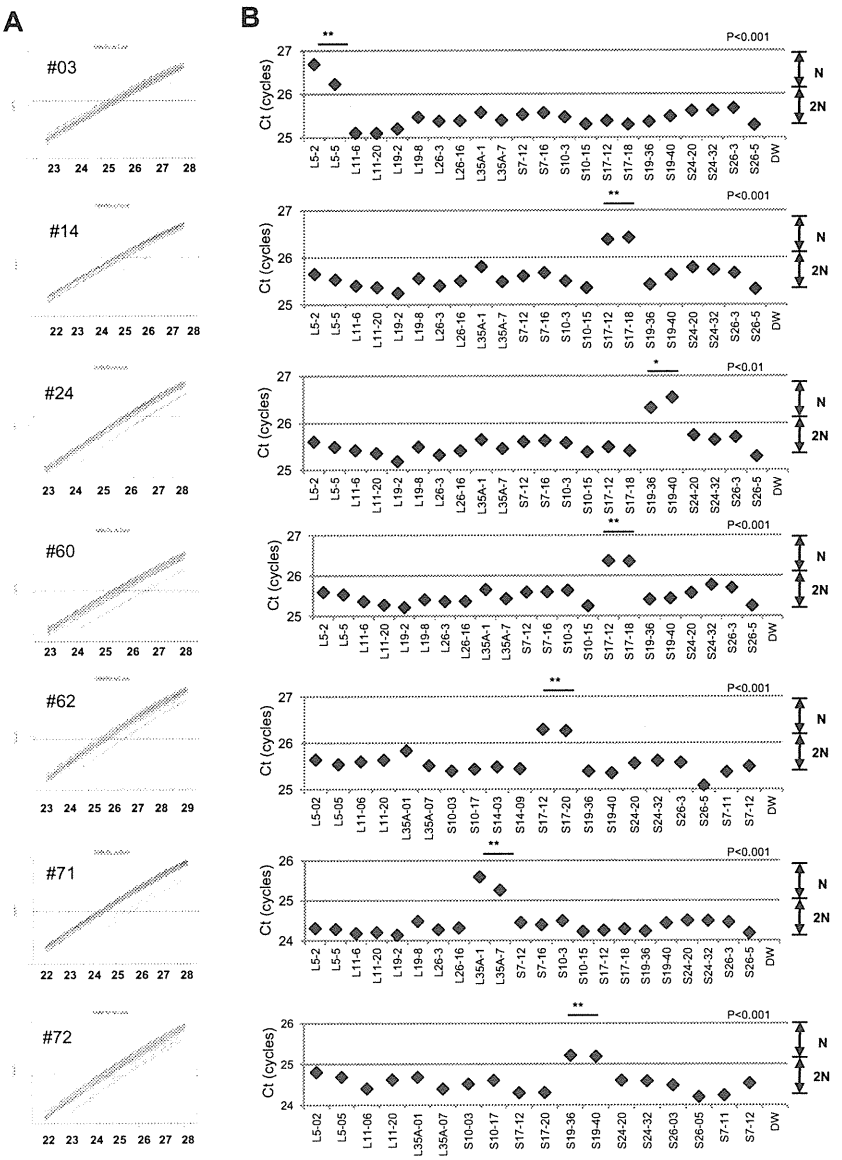

Figure 2. Detection of 7 mutations with a large deletion in DBA patients. Genomic DNA of 27 Japanese DBA patients with unknown mutations were subjected to the DBA gene copy number assay. (A) Amplification curve of s-q-PCR of a mutation with a large deletion. The deleted gene can be easily distinguished. (B) Ct score (cycles) of representative s-q-PCR with DBA genomic s-q-PCR primers. Results of the 2 gene-specific primer pairs indicated in the graph are representative of at least 2 sets for each gene-specific primer (carried out in the same run). $^{**}P < .001$; $^{*}P < .01$

chromosome 1 (ch1) spanning 858 kb (Figure 3A); patient 71 had a large deletion in ch3 spanning 786 kb (Figure 3B); patients 14, 60, and 62 had a large deletion in ch15 spanning 270 kb, 260 kb, and 330 kb, respectively (Figure 3C); and patient 72 had a large deletion in ch19 spanning 824 kb (Figure 3D). However, there were no deletions detected in ch19 in patient 24 (Figure 3D). Genes estimated to reside within a large deletion are listed in supplemental Table 1. Consistent with these s-q-PCR results, 6 of 7 large deletions were detected and confirmed as deleted regions, and these large deletions contained *RPL5*, *RPL35A*, *RPS17*, and *RPS19* (Table 4 and supplemental Table 1). Other large deletions in RP genes were not detected by this analysis. From these results, we conclude that the synchronized multiple PCR amplification method has a detection sensitivity comparable to that of SNP arrays.

Detailed examination of a patient with intragenic deletion in the RPS19 allele (patient 24)

Interestingly, for patient 24, in whom we could not detect a large deletion by SNP array at s-q-PCR gene copy number analysis, 2 primer sets for *RPS19* showed a 1-cycle delay (RPS19-36 and RPS19-40), but 2 other primer pairs (RPS19-58 and RPS19-62) did not show this delay (Figure 4A). We attempted to determine the deleted region in detail by testing more primer sets on *RPS19*. We tested a total of 9 primer sets for *RPS19* (Figure 4B) and examined the gene copy numbers. Surprisingly, 4 primer sets (S19-24, S19-36, S19-40, and S19-44) for intron 3 of *RPS19* indicated a 1-cycle delay, but the other primers for *RPS19* located on the 5′untranslated region (5′UTR), intron 3, or 3′UTR did not show this delay (S19-57, S19-58, S19-28, S19-62, and S19-65; Figure 4B-C). These results suggest that the intragenic deletion occurred in the *RPS19* allele. To confirm this deleted region precisely, we performed genomic PCR on *RPS19*, amplifying a region from the 5′UTR to intron 3 (Figure

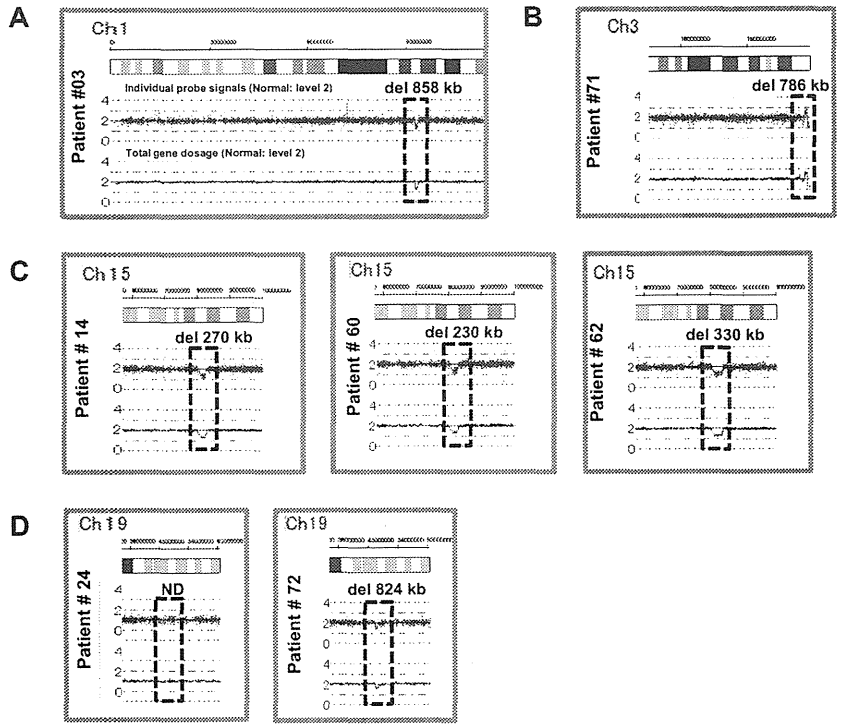

Figure 3. Results of SNP genomic microarray (SNP-chip) analysis. Genomic DNA of 27 Japanese DBA patients with unknown mutations was examined using a SNP array. Six patients had large deletions in their chromosome (ch), which included one DBA-responsible gene. Patient 3 has a large deletion in ch1 (A), patient 71 has a deletion in ch3 (B), patients 14, 60, and 62 have deletions in ch15 (C), and patient 72 has a deletion in ch19 (D).

4B). In patient 24, we observed an abnormally sized PCR product at a low molecular weight by agarose gel electrophoresis (Figure 4D). We did not detect a wild-type PCR product from the genomic PCR. This finding is probably because PCR tends to amplify smaller molecules more easily. However, we did detect a PCR fragment at the correct size using primers located in the supposedly deleted region. These bands were thought to be from the products of a wild-type allele. Sequencing of the mutant band revealed that intragenic recombination occurred at a homologous region of 27 nucleotides, from −1400 to −1374 in the 5′ region, to +5758 and +5784 in intron 3, which resulted in the loss of 7157 base pairs in the *RPS19* gene (Figure 4E). The deleted region contains exons 1, 2, and 3, and therefore the correct *RPS19* mRNA could not be transcribed.

Genotype-phenotype analysis and DBA mutations in Japan

Patients with a large deletion in DBA genes had common phenotypes (Table 4). Malformation with growth retardation (GR), including short stature or SGA, were observed in all 7 patients. In patients who had a mutation found by sequencing, half had GR (11 of 22; status data of DBA patients with mutations found by sequencing are not shown). GR may be a distinct phenotypic feature of large deletion mutations in Japanese DBA patients. Familial mutations were analyzed for parents for 5 DBA patients with a large deletion (patients 3, 24, 60, 62, and 72) by s-q-PCR. There are no large deletions in all 5 pairs of parents in DBA-responsible genes. Four of the 7 patients responded to steroid therapy. We have not observed significant phenotypic differences between patients with extensive deletions and other patients with regard to blood counts, responsiveness to treatment, or other malformations.

Discussion

Many studies have reported RP genes to be responsible for DBA. However, mutations have not been determined for approximately half of DBA patients analyzed. There are 2 possible reasons for this finding. One possibility is that patients have other genes responsible for DBA, and the other is that patients have a complicated set of mutations in RP genes that are difficult to detect. In the present study, we focused on the latter possibility because we have found fewer Japanese DBA patients with RP gene mutations (32.4%) compared with another cohort study of 117 DBA patients and 9 RP genes (approximately 52.9%).[4] With our newly developed method, we identified 7 new mutations with a large deletion in *RPL5*, *RPL35A*, *RPS17*, and *RPS19*.

The frequency of a large deletion was approximately 25.9% (7 of 27) in our group of patients who were not found to have mutations by genomic sequencing. Therefore, total RP gene mutations were confirmed in 42.6% of these Japanese patients (Table 5). Interestingly, mutations in *RPS17* have been observed at a high rate (5.9%) in Japan relative to that in other countries (1%).[5,15,16] Although the percentage of DBA mutations differs among different ethnic groups,[8,17-19] a certain portion of large deletions in DBA-responsible genes are likely to be determined in other countries by new strategies.

In the present study, we analyzed patient data to determine genotype-phenotype relations. To date, large deletions have been reported with RPS19 and RPL35A in DBA patients.[3,6,13] *RPS19* large deletions/translocations have been reported in 12 patients, and *RPL35A* large deletions have been reported in 2 patients.[19] GR in patients with a large deletion has been observed previously with *RPS19* translocations,[3,19-21] but it was not found in 2 patients with *RPL35A* deletion.[6] Interestingly, all of our patients with a large deletion had a phenotype

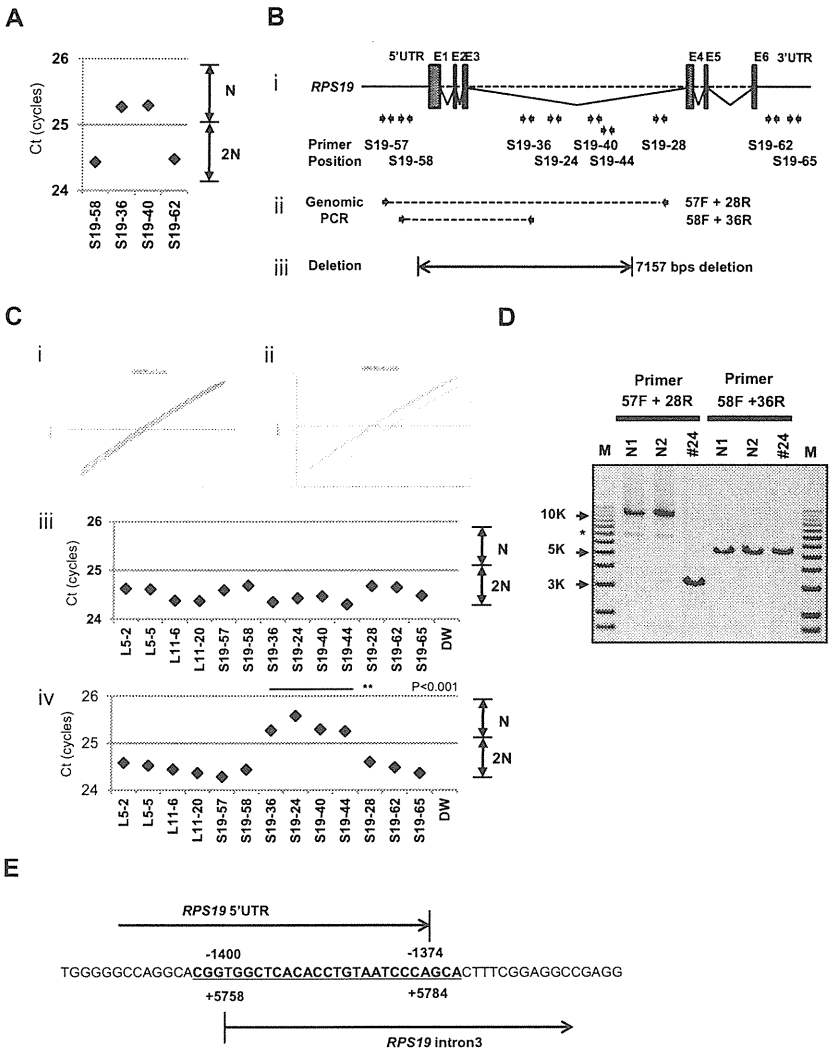

Figure 4. Result of s-q-PCR gene copy number assay for patient 24. (A) Results of s-q-PCR gene copy number assay for *RPS19* with 4 primer sets. (Bi) The *RPS19* gene copy number was analyzed with 9 specific primer sets for *RPS19* that span from the 5′UTR to the 3′UTR. (ii) Primer positions of genomic PCR for *RPS19*. (iii) Region determined to be an intragenic deletion in *RPS19*. (C) Results of gene copy number assay for *RPS19* show a healthy person (i,iii) and a DBA patient (ii,iv), and Ct results are shown (iii-iv). Patient 24 showed a "1-cycle delay" with primers located in the intron 3 region, but other primer sets were normal. (D) Results of genomic PCR amplification visualized by agarose gel electrophoresis to determine the region of deletion. N1 and N2 are healthy samples. *Nonspecific band. (E) Results from the genomic sequence of the 3-kb DNA band from genomic PCR on patient 24 showing an intragenic recombination from −1400 to 5784 (7157 nt) in *RPS19*. $^{**}P < .001$.

of GR, including short stature and SGA, which suggests that this is a characteristic of DBA with a large gene deletion in Japan. Our study results suggest the possibility that GR is associated with extensive deletion in Japanese patients. Although further case studies will be needed to confirm this possibility, screening of DBA samples using our newly developed method will help to advance our understanding of the broader implications of the mutations and the correlation with the DBA genotype-phenotype.

Table 5. Total mutations in Japanese DBA patients, including large gene deletions

Gene	Mutation rate
RPS19	12(17.6%)
RPL5	7(10.3%)
RPL11	3 (4.4%)
RPS17	4 (5.9%)
RPS10	1 (1.5%)
RPS26	1 (1.5%)
RPL35A	1 (1.5%)
RPS24	0
RPS14	0
Mutations, n (%)	29(42.6%)
Total analyzed, N	68

Copy number variation analysis of DBA has been performed by linkage analysis, and the *RPS19* gene was first identified as a DBA-susceptibility gene. Comparative genomic hybridization array technology has also been used to detect DBA mutations in *RPL35A*, and multiplex ligation-dependent probe amplification has been used for *RPS19* gene deletion analysis.[3,6,13,22] However, these analyzing systems have problems in mutation screening. Linkage analysis is not a convenient tool to screen for multiple genetic mutations, such as those in DBA, because it requires a high level of proficiency. Although comparative genomic hybridization technology is a powerful tool with which to analyze copy number comprehensively, this method requires highly specialized equipment and analyzing software, which limits accessibility for researchers. Whereas quantitative PCR–based methods for copy number variation analysis are commercially available (TaqMan), they require a standard curve for each primer set, which limits the number of genes that can be loaded on a PCR plate. To address this issue, a new method of analysis is needed. By stringent selection of PCR primers, the s-q-PCR method enables analysis of many DBA genes in 1 PCR plate and the ability to immediately distinguish a large deletion using the s-q-PCR amplification curve. In our study, 6 of 7 large deletions in the RP gene detected by s-q-PCR were confirmed by SNP arrays (Figure 3). Interestingly, we detected

1 large intragenic deletion in *RPS19*, which was not detected by the SNP array. This agreement between detection results suggests that the s-q-PCR copy number assay could be useful for detecting large RP gene deletions.

In the present study, 7 DBA patients carried a large deletion in the RP genes. This type of mutation could be underrepresented by sequencing analysis, although in the future, genome sequencing might provide a universal platform for mutation and deletion detection. We propose that gene copy number analysis for known DBA genes, in addition to direct sequencing, should be performed to search for a novel responsible gene for DBA. Although at present, it may be difficult to observe copy numbers on all 80 ribosomal protein genes in one s-q-PCR assay, our method allows execution of gene copy number assays for several target genes in 1 plate. Because our method is quick, easy, and low cost, it could become a conventional tool for detecting DBA mutations.

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Authorship

Contribution: M.K. designed and performed the research, analyzed the data, and wrote the manuscript; A.S-O. and S. Ogawa performed the SNP array analysis; T.M., M.T., and M.O designed the study; T.T, K. Terui, and R.W. analyzed the mutations and status data; H.K., S. Ohga, A.O., S.K., T.K., K.G., K.K., T.M., and N.M. analyzed the status data; A.M., H.M., K. Takizawa, T.M., and K.Y., performed the research and analyzed the data; E.I. and I.H. designed the study and analyzed the data; and all authors wrote the manuscript.

Conflict-of-interest disclosure: The authors declare no competing financial interests.

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References

1. Hamaguchi I, Flygare J, Nishiura H, et al. Proliferation deficiency of multipotent hematopoietic progenitors in ribosomal protein S19 (RPS19)-deficient diamond-Blackfan anemia improves following RPS19 gene transfer. *Mol Ther.* 2003;7(5 pt 1):613-622.
2. Vlachos A, Ball S, Dahl N, et al. Diagnosing and treating Diamond Blackfan anaemia: results of an international clinical consensus conference. *Br J Haematol.* 2008;142(6):859-876.
3. Draptchinskaia N, Gustavsson P, Andersson B, et al. The gene encoding ribosomal protein S19 is mutated in Diamond-Blackfan anaemia. *Nat Genet.* 1999;21(2):169-175.
4. Doherty L, Sheen MR, Vlachos A, et al. Ribosomal protein genes RPS10 and RPS26 are commonly mutated in Diamond-Blackfan anemia. *Am J Hum Genet.* 2010;86(2):222-228.
5. Gazda HT, Sheen MR, Vlachos A, et al. Ribosomal protein L5 and L11 mutations are associated with cleft palate and abnormal thumbs in Diamond-Blackfan anemia patients. *Am J Hum Genet.* 2008;83(6):769-780.
6. Farrar JE, Nater M, Caywood E, et al. Abnormalities of the large ribosomal subunit protein, Rpl35a, in Diamond-Blackfan anemia. *Blood.* 2008;112(5):1582-1592.
7. Gazda HT, Grabowska A, Merida-Long LB, et al. Ribosomal protein S24 gene is mutated in Diamond-Blackfan anemia. *Am J Hum Genet.* 2006;79(6):1110-1118.
8. Konno Y, Toki T, Tandai S, et al. Mutations in the ribosomal protein genes in Japanese patients with Diamond-Blackfan anemia. *Haematologica.* 2010;95(8):1293-1299.
9. Robledo S, Idol RA, Crimmins DL, Ladenson JH, Mason PJ, Bessler M. The role of human ribosomal proteins in the maturation of rRNA and ribosome production. *RNA.* 2008;14(9):1918-1929.
10. Léger-Silvestre I, Caffrey JM, Dawaliby R, et al. Specific Role for Yeast Homologs of the Diamond Blackfan Anemia-associated Rps19 Protein in Ribosome Synthesis. *J Biol Chem.* 2005;280(46):38177-38185.
11. Choesmel V, Fribourg S, Aguissa-Toure AH, et al. Mutation of ribosomal protein RPS24 in Diamond-Blackfan anemia results in a ribosome biogenesis disorder. *Hum Mol Genet.* 2008;17(9):1253-1263.
12. Flygare J, Aspesi A, Bailey JC, et al. Human RPS19, the gene mutated in Diamond-Blackfan anemia, encodes a ribosomal protein required for the maturation of 40S ribosomal subunits. *Blood.* 2007;109(3):980-986.
13. Quarello P, Garelli E, Brusco A, et al. Multiplex ligation-dependent probe amplification enhances molecular diagnosis of Diamond-Blackfan anemia due to RPS19 deficiency. *Haematologica.* 2008;93(11):1748-1750.
14. Yamamoto G, Nannya Y, Kato M, et al. Highly sensitive method for genomewide detection of allelic composition in nonpaired, primary tumor specimens by use of affymetrix single-nucleotide-polymorphism genotyping microarrays. *Am J Hum Genet.* 2007;81(1):114-126.
15. Song MJ, Yoo EH, Lee KO, et al. A novel initiation codon mutation in the ribosomal protein S17 gene (RPS17) in a patient with Diamond-Blackfan anemia. *Pediatr Blood Cancer.* 2010;54(4):629-631.
16. Cmejla R, Cmejlova J, Handrkova H, Petrak J, Pospisilova D. Ribosomal protein S17 gene (RPS17) is mutated in Diamond-Blackfan anemia. *Hum Mutat.* 2007;28(12):1178-1182.
17. Cmejla R, Cmejlova J, Handrkova H, et al. Identification of mutations in the ribosomal protein L5 (RPL5) and ribosomal protein L11 (RPL11) genes in Czech patients with Diamond-Blackfan anemia. *Hum Mutat.* 2009;30(3):321-327.
18. Quarello P, Garelli E, Carando A, et al. Diamond-Blackfan anemia: genotype-phenotype correlations in Italian patients with RPL5 and RPL11 mutations. *Haematologica.* 2010;95(2):206-213.
19. Boria I, Garelli E, Gazda HT, et al. The ribosomal basis of Diamond-Blackfan Anemia: mutation and database update. *Hum Mutat.* 2010;31(12):1269-1279.
20. Campagnoli MF, Garelli E, Quarello P, et al. Molecular basis of Diamond-Blackfan anemia: new findings from the Italian registry and a review of the literature. *Haematologica.* 2004;89(4):480-489.
21. Willig TN, Draptchinskaia N, Dianzani I, et al. Mutations in ribosomal protein S19 gene and diamond blackfan anemia: wide variations in phenotypic expression. *Blood.* 1999;94(12):4294-4306.
22. Gustavsson P, Garelli E, Draptchinskaia N, et al. Identification of microdeletions spanning the Diamond-Blackfan anemia locus on 19q13 and evidence for genetic heterogeneity. *Am J Hum Genet.* 1998;63(5):1388-1395.

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*

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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

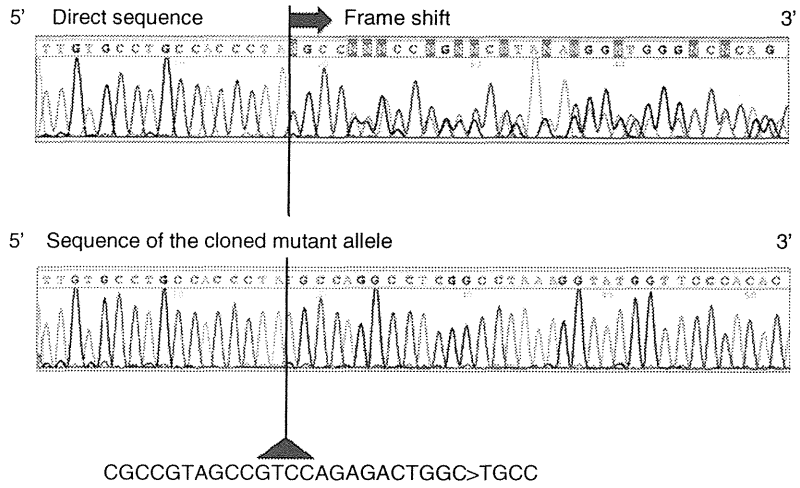

FIGURE 1. Sequence changes and frameshift in the *RPS10*. Direct sequencing showed a separated signal derived from the heteroduplex polymerase chain reaction product from the third exon of *RPS10*. Sequencing of the cloned mutant allele identified a heterozygous mutation (c.283_306delinsTGCC) and frameshift.

(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

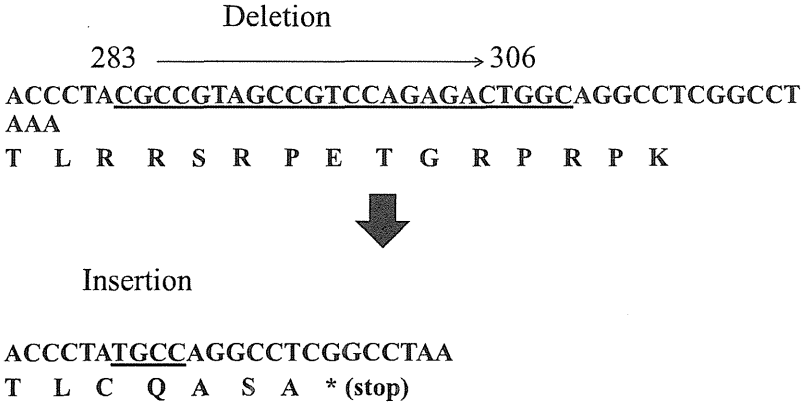

FIGURE 2. Deletion and insertion of this patient in *RSP10*. The c.283_306delinsTGCC mutation resulted in a frameshift at codon 95 and a "stop" at codon 100.

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.

REFERENCES

1. Vlachos A, Ball S, Dahl N, et al. Diagnosing and treating Diamond Blackfan anaemia: results of an international clinical consensus conference. *Br J Haematol.* 2008;142:859–876.
2. Gazda HT, Sieff CA. Recent insights into the pathogenesis of Diamond-Blackfan anemia. *Br J Haematol.* 2006;135:149–157.
3. Flygare J, Karlsson S. Diamond-Blackfan anemia: erythropoiesis lost in translation. *Blood.* 2007;109:3152–3160.
4. Draptchinskaia N, Gustavsson P, Andersson B, et al. The gene encoding ribosomal protein S19 is mutated in Diamond-Blackfan anemia. *Nat Genet.* 1999;21:169–175.
5. Gazda HT, Grabowska A, Merida-Long LB, et al. Ribosomal protein S24 gene is mutated in Diamond-Blackfan anemia. *Am J Hum Genet.* 2006;79:1110–1118.
6. Cmejla R, Cmejlova J, Handrkowa H, et al. Ribosomal protein S17 gene (RPS17) is mutated in Diamond-Blackfan anemia. *Hum Mutat.* 2007;28:1178–1182.
7. Ito E, Konno Y, Toki T, Terui K. Molecular pathogenesis in Diamond-Blackfan anemia. *Int J Hematol.* 2010;92:413–418.
8. Doherty L, Sheen MR, Vlachos A, et al. Ribosomal protein genes RPS10 and RPS26 are commonly mutated in Diamond-Blackfan anemia. *Am J Hum Genet.* 2010;86:222–228.
9. Konno Y, Toki T, Tandai S, et al. Mutations in the ribosomal protein genes in Japanese patients with Diamond-Blackfan anemia. *Haematologica.* 2010;95:1293–1299.
10. Lēger-Silvestre I, Caffrey JM, Dawaliby R, et al. Specific role for yeast homologs of the Diamond Blackfan anemia-associated RPS19 protein in ribosome synthesis. *J Biol Chem.* 2005;280:38177–38185.
11. Choesmel V, Bacquevulle D, Rouquette J, et al. Impaired ribosome biogenesis in Diamond-Blackfan anemia. *Blood.* 2007;109:1275–1283.
12. Flygare J, Aspesi A, Bailey JC, et al. Human RPS19, the gene mutation in Diamond-Blackfan anemia, encodes a ribosomal protein required for the maturation of 40S ribosomal subunits. *Blood.* 2007;109:980–986.
13. Idol RA, Robledo S, Du HY, et al. Cells depleted for RPS19, protein associated in Diamond-Blackfan anemia, show defects in 18S ribosomal RNA synthesis and small ribosomal subunit production. *Blood Cell Mol Dis.* 2007;39:35–43.
14. Farrar JE, Nater M, Caywood E, et al. Abnormalities of the large ribosomal subunit protein, Rpl35a, in Diamond-Blackfan anemia. *Blood.* 2008;112:1582–1592.
15. Gazda HT, Sheen MR, Vlachos A, et al. Ribosomal Protein L5 and L11 mutations are associated with cleft palate and abnormal thumbs in Diamond-Blackfan anemia patients. *Am J Hum Genet.* 2008;83:769–780.
16. Choesmel V, Fribourg S, Aguissa-Tourē AH, et al. Mutation of ribosomal protein RPS24 in Diamond-Blackfan anemia results in ribosome biogenesis disorder. *Hum Mol Genet.* 2008;17:1253–1263.
17. Perdahl EB, Naprstek BI, Wallace WC, et al. Erythroid failure in Diamond-Blackfan anemia is characterized by apoptosis. *Blood.* 1994;83:645–650.
18. Miyake K, Utsugisawa T, Flygare J, et al. Ribosomal protein S19 deficiency leads to reduced proliferation and increased apoptosis but does not affect terminal erythroid differentiation in a cell line model of Diamond-Blackfan anemia patients. *Stem Cells.* 2008;26:323–329.
19. Danilova N, Sakamoto KM, Lin S. Ribosomal protein S19 deficiency in zebrafish leads to developmental abnormalities and defective erythropoiesis through activation of p53 protein family. *Blood.* 2008;112:5228–5237.
20. McGowan KA, Li JZ, Park CY, et al. Ribosomal mutations cause p53-mediated dark shin and pleiotropic effect. *Nat Genet.* 2008;40:963–970.

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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ゾ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