

### Ⅲ. 研究成果の刊行に関する一覧表

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◎は、本研究によることが明記されている論文

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書籍

著者氏名	論文タイトル名	書籍全体の編集者名	書籍名	出版社名	出版地	出版年	ページ
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## IV. 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.<sup>1</sup> 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.<sup>2</sup>

Mutations in the *RPS19* gene were first reported in 25% of DBA patients by Drapchinskaia et al in 1999.<sup>3</sup> 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%).<sup>4-7</sup> 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.<sup>8</sup> 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., unpub-

lished 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,<sup>4-6,9-12</sup> 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*.<sup>3,6,13</sup> 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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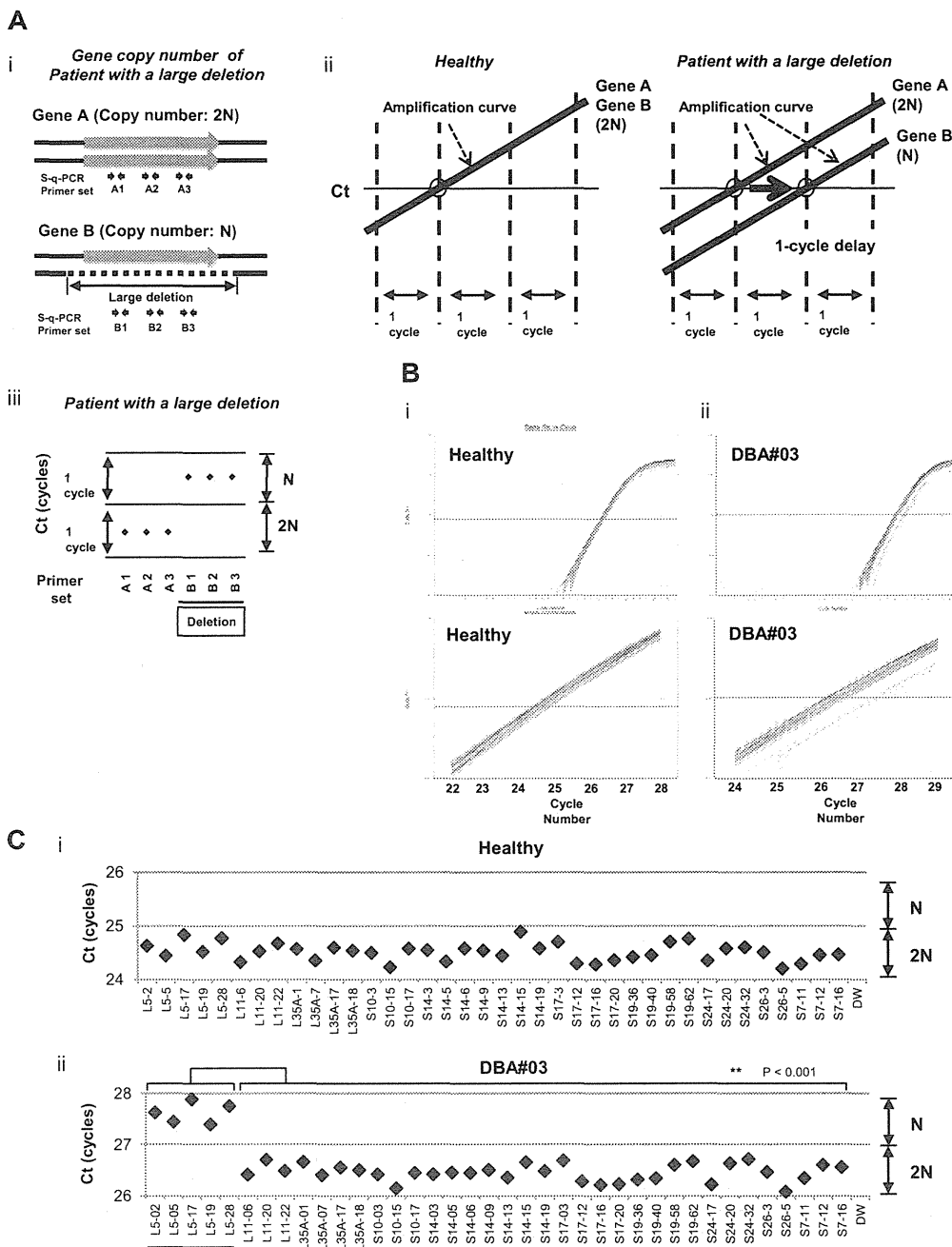
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Table 1. Primers used for synchronized quantitative-PCR (s-q-PCR) of RPL proteins

Gene	Primer name	Sequence	Primer name	Sequence	Size, bp
RPL5	L5-02F	CTCCCAAAGTGCTTGAGATTACAG	L5-02R	CACCTTTTCTAACAAATTCCTCAAT	132
	L5-05F	AGCCCTCCAACCTAGGTGACA	L5-05R	GAATTGGGATGGGCAAGAACT	102
	L5-17F	TGAACCCCTTGCCTAAACATG	L5-17R	TCTTGGTCAGGCCCTGCTTA	105
	L5-19F	ATTGTGCAAACTCGATCACTAGCT	L5-19R	GTGTCTGAGGCTAACACATTTCAT	103
	L5-21F	GTGCCACTCTCTTGGACAACTG	L5-21R	CATAGGGCCAAAAGTCAAATAGAAG	102
	L5-28F	TCCACTTTAGGTAGGCGAAACC	L5-28R	TCAGATTTGGCATGTACCTTTCA	102
RPL11	L11-06F	GCACCCACATGGCTTAAAGG	L11-6R	CAACCAACCCATAGGCCAAA	102
	L11-20F	GAGCCCTTTTCTCAGATGATA	L11-20R	CATGAACCTTGGGCTCTGAATCC	109
	L11-22F	TATGTGCAGATAAGAGGGCAGTCT	L11-22R	ATACAGATAAGGAACTGAGGCAGATT	98
RPL19	L19-02F	TGGCTCTCATAAAGGAAATCTCT	L19-02R	GGAATGCAGGCAAGTTACTCTGTT	103
	L19-08F	TTTGAAGGCAAGAAATAAGTTCCA	L19-08R	AGCACATCACAGAGTCCAAATAGG	107
	L19-16F	GGTTAGTTGAAGCAGGAGCCTTT	L19-16R	TGCTAGGGAGACAGAAGCACATC	102
RPL26	L19-19F	GGACCAGTAGTTGTGACATCAGTTAAG	L19-19R	CCCATTGTAAACCCCACTTG	106
	L26-03F	TCCAAAGAGCTGAGACAGAAGTACA	L26-03R	TCCATCAAGACAACGAGAACAAGT	102
	L26-16F	TTTGAGAATGCTTGAGAGAAGGAA	L26-16R	TTCCAGCACATGTAAATCAAGGA	102
	L26-18F	ATGTTTTAATAAGCCCTCCAGTTGA	L26-18R	GAGAACAGCAAGTTGAAAGGTTCA	102
RPL35A	L26-20F	GGGCTTTGCTTGATCACTCTAGA	L26-20R	AGGGAGCCCGAAAACATTTAC	104
	L35A-01F	TGTGGCTTCTATTTTGCCTCAT	L35A-01R	GGAATTACCTCCTTTATTGCTTACAAG	121
	L35A-07F	TTTCCGTTCTGTCTATTGCTGTGT	L35A-07R	GAACCTGAGTGGAGGATGTTCC	113
	L35A-17F	GCCCAACACCTCCAGAGAATC	L35A-17R	GGATCACTTGAGCGACGAAT	104
RPL36	L35A-18F	TTAGGTGGGCTTTTCACTCTCAA	L35A-18R	ATCTCCTGATTCCTCACTTTGT	102
	L36-02F	CCGCTCTACAAGTGAAGAAATCTG	L36-02R	CTCCCTCTGCCTGTGAAATGA	102
	L36-04F	TGCGTCTGCCAGTGTGG	L36-04R	GGGTAGCTGTGAGAACAAGGT	105
	L36-17F	CCCCTTGAAGGACAGCAGTT	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	GAACCATGCAAAAAGTGCCAATAT	112
RPS10	S10-03F	CTACGGTTTTGTGTGGGCTCACTT	S10-03R	CATCTGCAAGAAGGAGACGATTG	102
	S10-15F	GTTGGCCCTGGAGTCTGATTT	S10-15R	ATTCCAAGTGCACCATTTCCTT	101
	S10-17F	AATGGTGTITAGGCCAACGTTAC	S10-17R	TTTGAACAGTGGTTTTGTGCAT	100
RPS14	S14-03F	GAATTCCAAACCTTCTGCAAA	S14-03R	TTGCTTCAITTTACTCCTCAAGACATT	104
	S14-05F	ACAACCAACCCCTTACCTCTTTT	S14-05R	GGAAGACGCCGCATTATT	102
	S14-06F	CGCCTCTACCTCGCCAAAC	S14-06R	GGGATCGGTGCTATTGTTATTCC	102
	S14-09F	GCCATCATGCCGAAACATACT	S14-09R	AACGCGCCACAGGAGAGA	102
	S14-13F	ATCAGGTGGAGCACAGGAAAC	S14-13R	GCGAGGGAGCTCTTGATT	111
	S14-15F	AGAAAGTTTTAGTGAGGCAGAAATGAGA	S14-15R	TCCCTTGCTATTAAATGAAACC	102
	S14-19F	GATGAATGTCTTTCTCCATTC	S14-19R	TAGGCGGAAACCAAAAATGCT	102
RPS15	S15-11F	CTCAGCTAATAAAGCGCACATG	S15-11R	CCTCACACCACGAACCTGAAG	108
	S15-15F	GGTTGGAGAACATGGTGAGAACTA	S15-15R	CACATCCCTGGGCCACTCT	108
RPS17	S17-03F	ACTGCTGTCTGCTGGCTGATT	S17-03R	GATGACCTGTCTTCTTGGCCTTA	121
	S17-05F	GAAAACAGATACAAATGGCATGGT	S17-05R	TGCCTCCCCTTTTCCAGAGT	114
	S17-12F	CTATGTGTAGGAGGTCAGGATAG	S17-12R	CCACCTGGTACTGAGCACATGT	102
	S17-16F	TAGCGGAAGTTGTGTGCATTG	S17-16R	CAAGAACAAGAGCAGCCAAAGG	102
	S17-18F	TGGCTGAATCTGCCTGCTT	S17-18R	GCCTTGATGTACCTGGAATGG	103
	S17-20F	GGGCCCTTCAAAATGTTGA	S17-20R	GCAAAACTCTGTCCCTTTGAGAA	101
RPS19	S19-24F	CCATCCCAAGAATGCACACA	S19-24R	CGCCGTAGCTGGTACTCATG	120
	S19-28F	GACACACCTGTTGAGTCCCTCAGAGT	S19-28R	GCTTCTATTAACCTGGAGCACACATCT	114
	S19-36F	CTCTTGAGGGTGGCTTGAAAT	S19-36R	GTCTTTGCGGTCTTCCCTTAC	102
	S19-40F	GGAACGGTGTGAGGATTCAGG	S19-40R	AGCGGCTGTACACCAGAAATG	101
	S19-44F	CTGAGGTTGAGTGTCCATTTCT	S19-44R	GCACCGGGCTCTGTTATC	104
	S19-57F	CAGGGACACAGTGTGAGAAACT	S19-57R	TGAGATGTCCATTTTCACTATTGTT	101
	S19-58F	CATGATGTTAGTCCGTTGCATA	S19-58R	ATTTTGGGAAGAGTGAAGCTTAGGT	102
	S19-62F	GCAACAGAGCGAGACTCCATTT	S19-62R	AGCACTTTTGGCCTTACTTCA	102
	S19-65F	ACATTTCCAGAGCTGACATGA	S19-65R	TCGGGACACCTAGACCTTGCT	102
RPS24	S24-17F	CGACCACGCTGGCTTAGAGT	S24-17R	CCTTCATGCCCAACCAAGTC	101
	S24-20F	ACAAGTAAGCATCATCACTCGAA	S24-20R	TTTCCCTCACAGCTATCGTATGG	105
	S24-32F	GGGAAATGCTGTGCCACATACT	S24-32R	CTGGTTTCATGGCTCCAGAGA	105
RPS26	S26-03F	CGCAGCAGTCAGGACATTT	S26-03R	AAGTTGGGCGAAGGCTTTAAG	104
	S26-05F	ATGGAGGCCGTCTAGTTGGT	S26-05R	TGCCTACCCTGAACCTTGCT	102
RPS27A	S27A-09F	GCTGGAGTGCATTGCTTGT	S27A-09R	CACGCCTGTAATCCCACTAA	102
	S27A-12F	CAGGCTTGGTGTGCTGTGACT	S27A-12R	ACGTCCATCTTCCAGCTGCTT	103
	S27A-18F	GGGTTTTCTGTTTGGTATTTGA	S27A-18R	AAAGGCCAGCTTTGCAAGTG	111
	S27A-22F	TTACCATATTGCCAGTCTTTCCATT	S27A-22R	TTCATATGCATTTGCACAACTGT	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.

tation of patients from a Japanese DBA genomic library are listed elsewhere or are as reported by Konno et al.<sup>8</sup> The study was approved by the institutional review board at the National Institute of Infectious Diseases and Hirosaki University.

**Methods**

**Patient samples**

Genomic DNA was extracted using the GenElute Blood Genomic DNA Kit (Sigma-Aldrich) according to the manufacturer’s protocol. Clinical manifes-

**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/gen/>) 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.<sup>14</sup> All microarray data are available at the EGA database ([www.ebi.ac.uk/ega](http://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

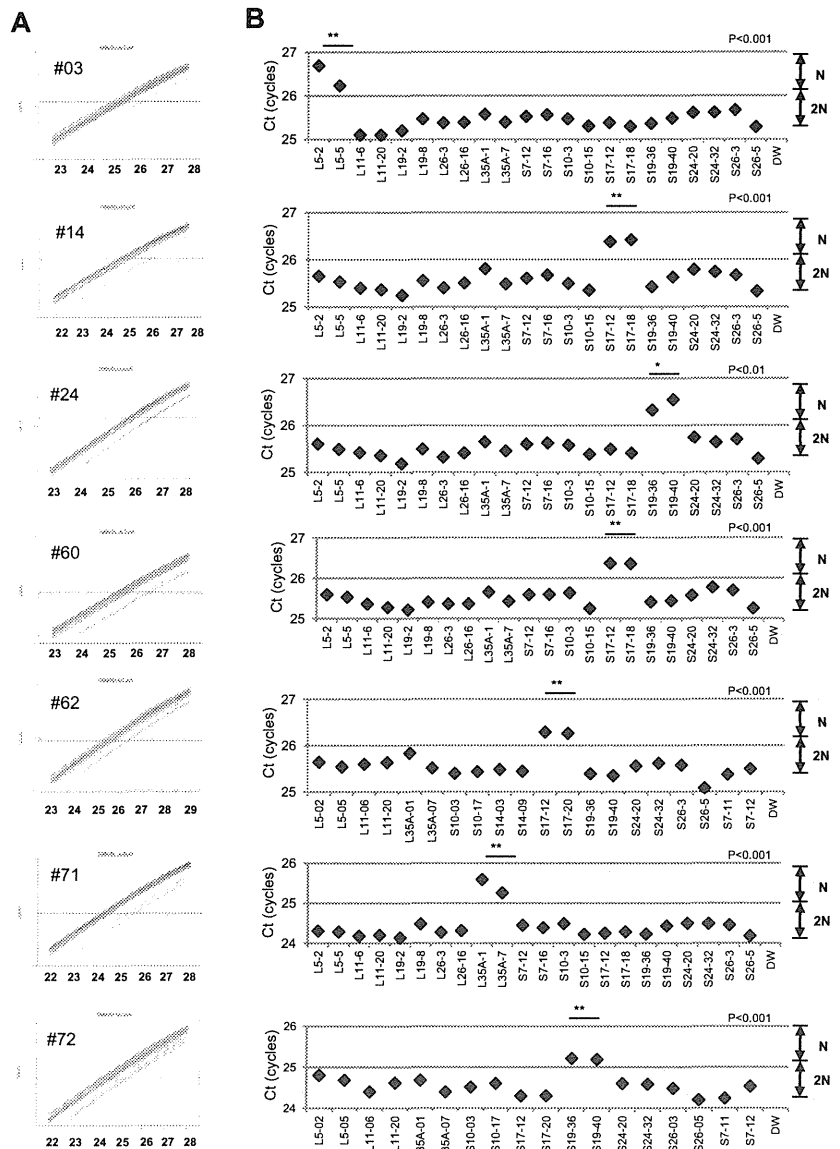
Patient no.	Age at diagnosis	Sex	Hb, g/dL	Large deletion by s-q-PCR	Large deletion by SNP array	Inheritance	Malformations	Response to first steroid therapy
<b>Patients with a large deletion in RP genes</b>								
3*†	1 y	M		RPL5	RPL5	Sporadic	Short stature, thumb anomalies	Response
14*	5 y	M	5.5	RPS17	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
<b>Patients without a large deletion in RP genes</b>								
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.<sup>8</sup>

†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). \*\* $P < .001$ ; \* $P < .01$

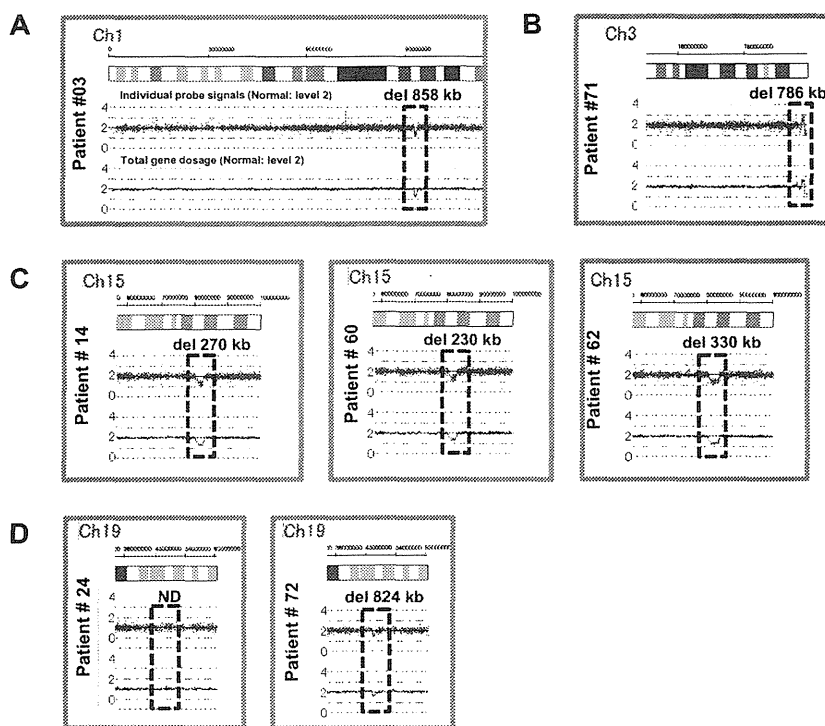


chromosome 1 (ch1) spanning 858 kb (Figure 3A); patient 71 had a large deletion in ch3 spanning 786 kb (Figure 3B); patients 14, 60, and 62 had a large deletion in ch15 spanning 270 kb, 260 kb, and 330 kb, respectively (Figure 3C); and patient 72 had a large deletion in ch19 spanning 824 kb (Figure 3D). 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.

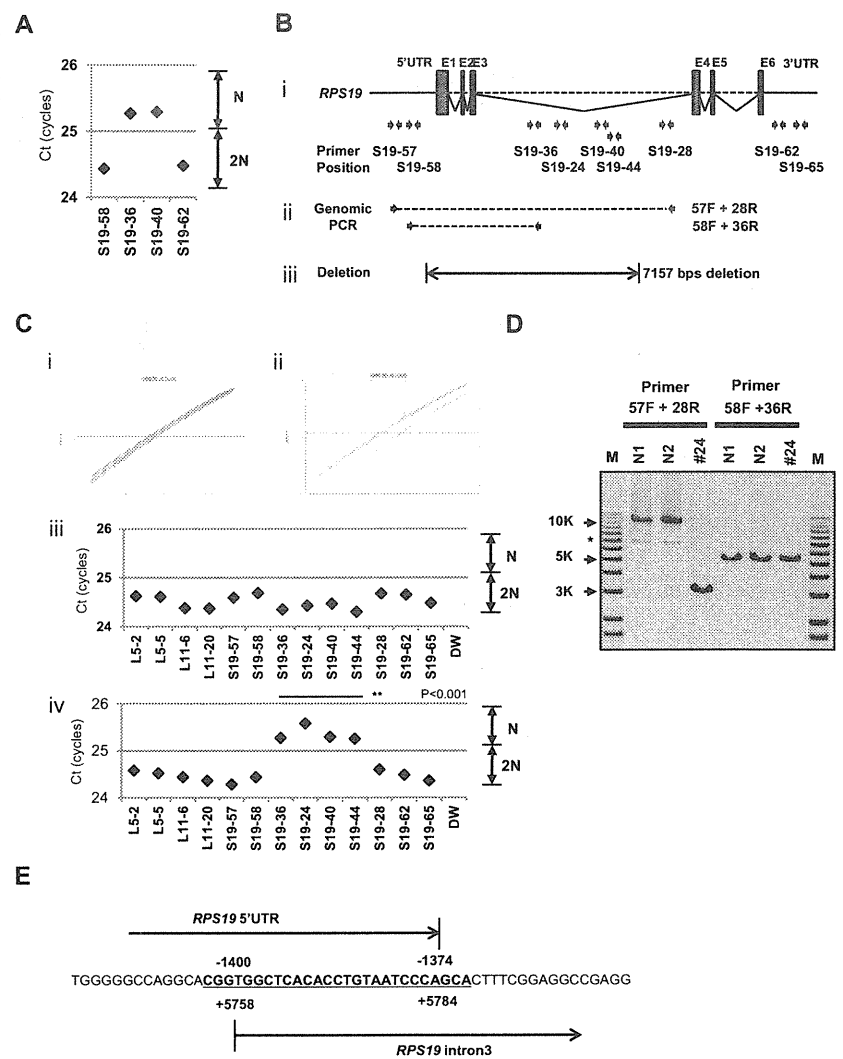
## Discussion

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%).<sup>4</sup> 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%).<sup>5,15,16</sup> Although the percentage of DBA mutations differs among different ethnic groups,<sup>8,17-19</sup> 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.<sup>3,6,13</sup> *RPS19* large deletions/translocations have been reported in 12 patients, and *RPL35A* large deletions have been reported in 2 patients.<sup>19</sup> GR in patients with a large deletion has been observed previously with *RPS19* translocations,<sup>3,19-21</sup> but it was not found in 2 patients with *RPL35A* deletion.<sup>6</sup> 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. (B) 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.

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.<sup>3,6,13,22</sup> 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

**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

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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## To the editor:

**Rabbit antithymocyte globulin and cyclosporine as first-line therapy for children with acquired aplastic anemia**

Horse antithymocyte globulin (hATG) and cyclosporine have been used as standard therapy for children with acquired aplastic anemia (AA) for whom an HLA-matched family donor is unavailable. However, in 2009, hATG (lymphoglobulin; Genzyme) was withdrawn and replaced by rabbit ATG (rATG; thymoglobulin; Genzyme) in Japan. Many other countries in Europe and Asia are facing the same situation.<sup>1</sup> Marsh et al recently reported outcomes for 35 adult patients with AA who were treated with rATG and cyclosporine as a first-line therapy.<sup>2</sup> Although the hematologic response rate was 40% at 6 months, several patients subsequently achieved late responses. The best response rate was 60% compared with 67% in a matched-pair control group of 105 patients treated with hATG. The overall and transplantation-free survival rates appeared to be significantly inferior with rATG compared with hATG at 68% versus 86% ( $P = .009$ ) and 52% versus 76% ( $P = .002$ ), respectively. These results are comparable to those from a prospective randomized study reported by Scheinberg et al comparing hATG and rATG.<sup>3</sup> Both studies showed the superiority of hATG over rATG.<sup>2,3</sup>

We recently analyzed outcomes for 40 Japanese children (median age, 9 years; range, 1-15) with AA treated using rATG and cyclosporine. The median interval from diagnosis to treatment was 22 days (range, 1-203). The numbers of patients with very severe, severe, and nonsevere disease were 14, 10, and 16, respectively. The ATG dose was 3.5 mg/kg/day for 5 days. The median follow-up time for all patients was 22 months (range, 6-38). At 3 months, no patients had achieved a complete response (CR) and partial response (PR) was seen in only 8 patients (20.0%). At 6 months, the numbers of patients with CR and PR were 2 (5.0%) and 17 (42.5%), respectively. After 6 months, 5 patients with PR at 6 months had achieved CR and 4 patients with no response at 6 months had achieved PR, offering a total best response rate of 57.5%. Two patients relapsed at 16 and 19 months without receiving any second-line treatments. Two patients with no re-

sponse received a second course of rATG at 13 and 17 months, but neither responded. Sixteen patients underwent hematopoietic stem cell transplantation (HSCT) from alternative donors (HLA-matched unrelated donors,  $n = 13$ ; HLA-mismatched family donors,  $n = 3$ ). Two deaths occurred after rATG therapy, but no patients died after HSCT. Causes of death were intracranial hemorrhage at 6 months and acute respiratory distress syndrome at 17 months. The overall 2-year survival rate was 93.8% and the 2-year transplantation-free survival rate was 50.3% (Figure 1).

In our previous prospective studies with hATG, the response rates after 6 months were 68% and 70%, respectively, with no increases in response rates observed after 6 months.<sup>4,5</sup> Our results support the notion that rATG is inferior to hATG for the treatment of AA in children. First-line HSCT from an alternative donor may be justified, considering the excellent outcomes in children who received salvage therapies using alternative donor HSCT.

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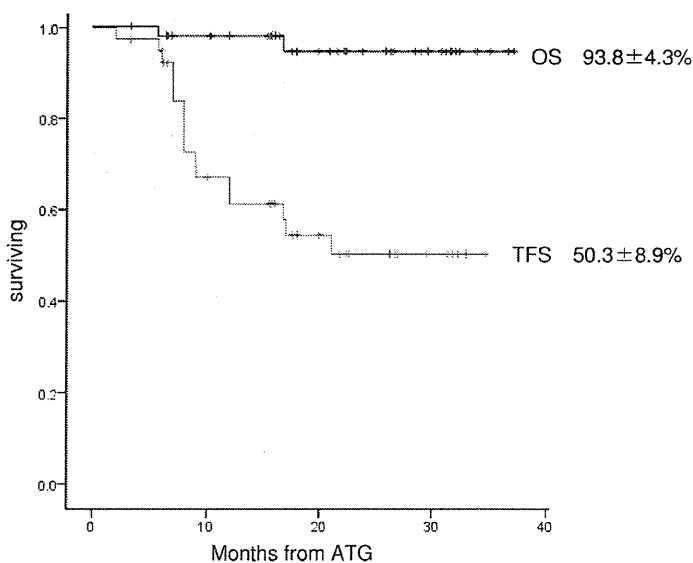
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**Figure 1.** Kaplan-Meier estimates of overall survival (OS) and transplantation-free survival (TFS) in 40 Japanese children with AA. Survival was investigated using Kaplan-Meier methods. OS for all patients with AA after rATG and cyclosporine as first-line therapy included patients who later received HSCT for nonresponse to rATG. In the analysis of TFS for all patients treated with rATG and CSA, transplantation was considered an event.