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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

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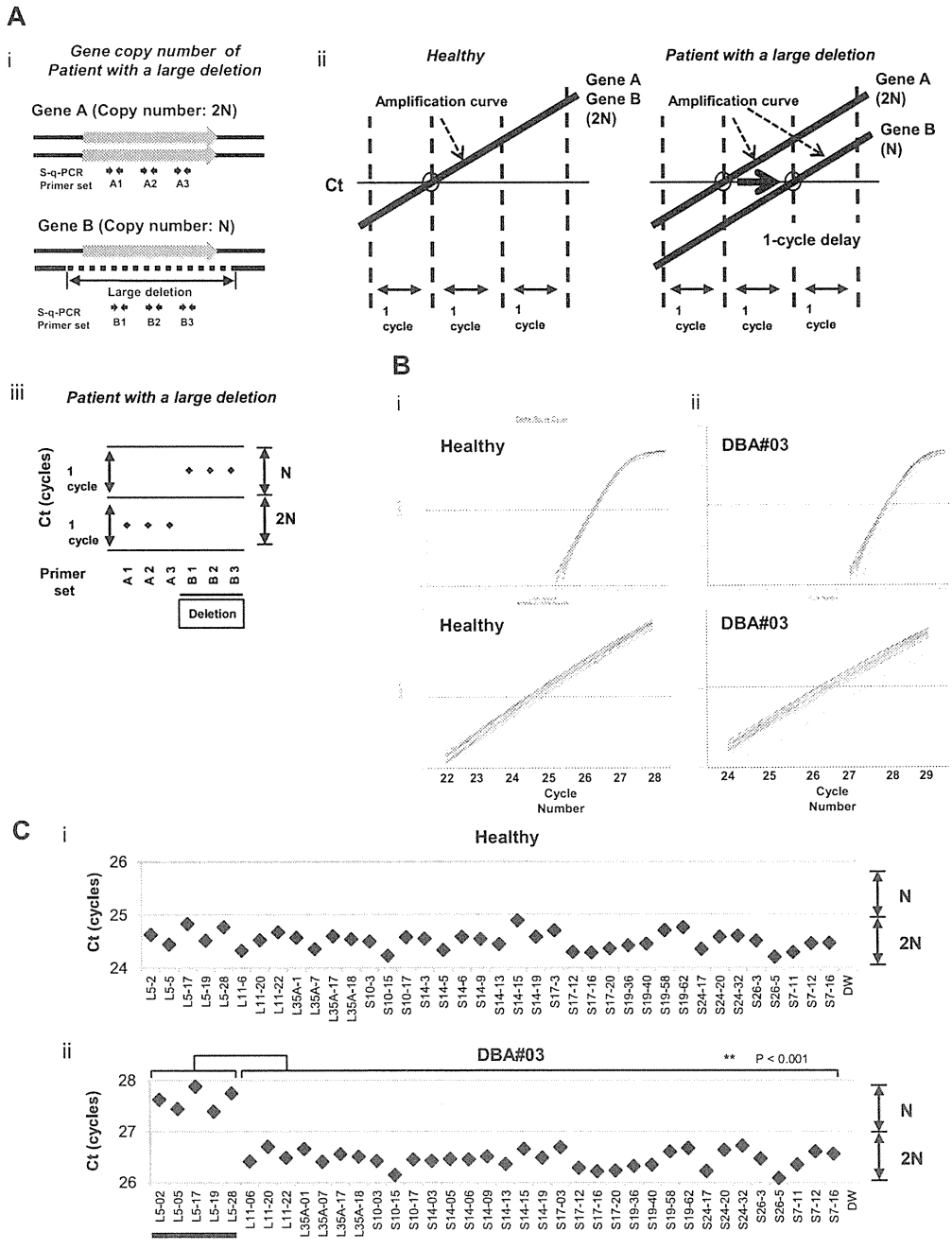

Figure 1. s-q-PCR can determine a large gene deletion in DBA. (A) Concept of the DBA s-q-PCR assay. The difference in gene copy number between a healthy sample and that with a large deletion is 2-fold (i). When all genomic s-q-PCR for genes of interest synchronously amplify DNA fragments, a 2-fold difference 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

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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

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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.[8]

†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.

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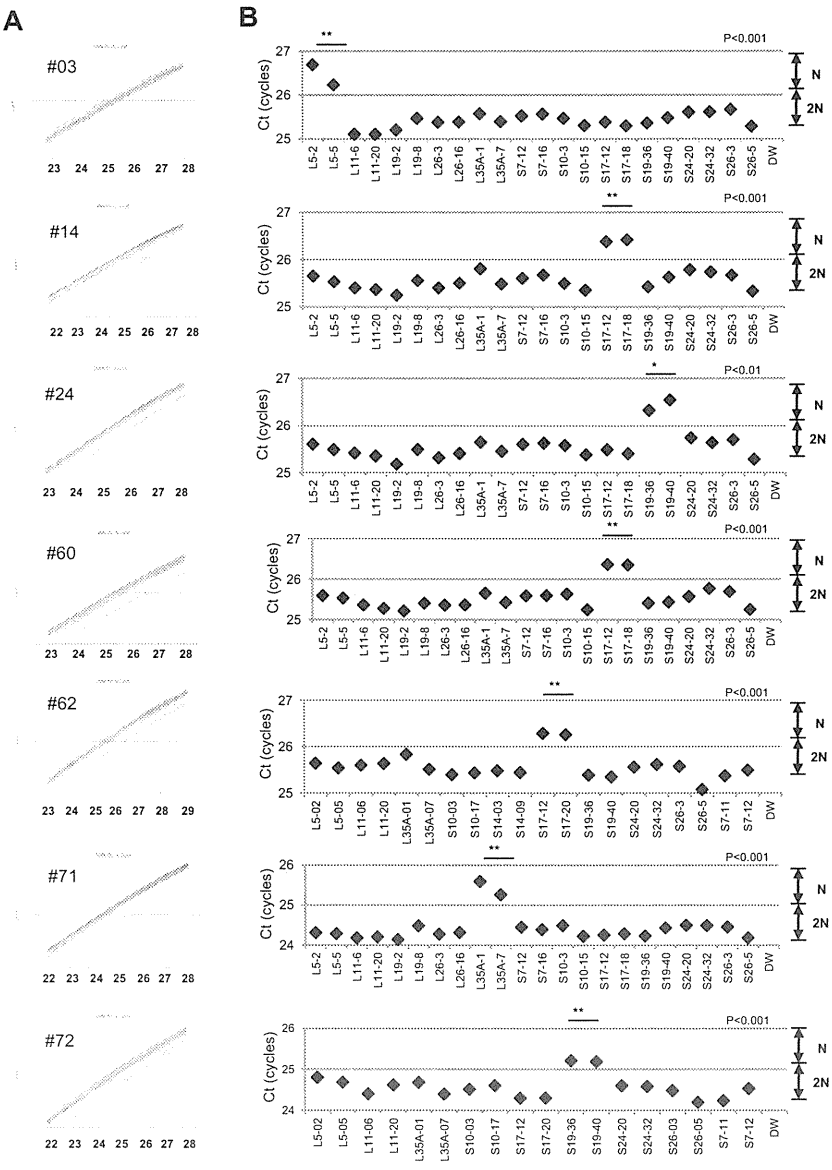

Figure 2. Detection of 7 mutations with a large deletion in DBA patients. Genomic DNA of 27 Japanese DBA patients with unknown mutations were subjected to the DBA gene copy number assay. (A) Amplification curve of s-q-PCR of a mutation with a large deletion. The deleted gene can be easily distinguished. (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

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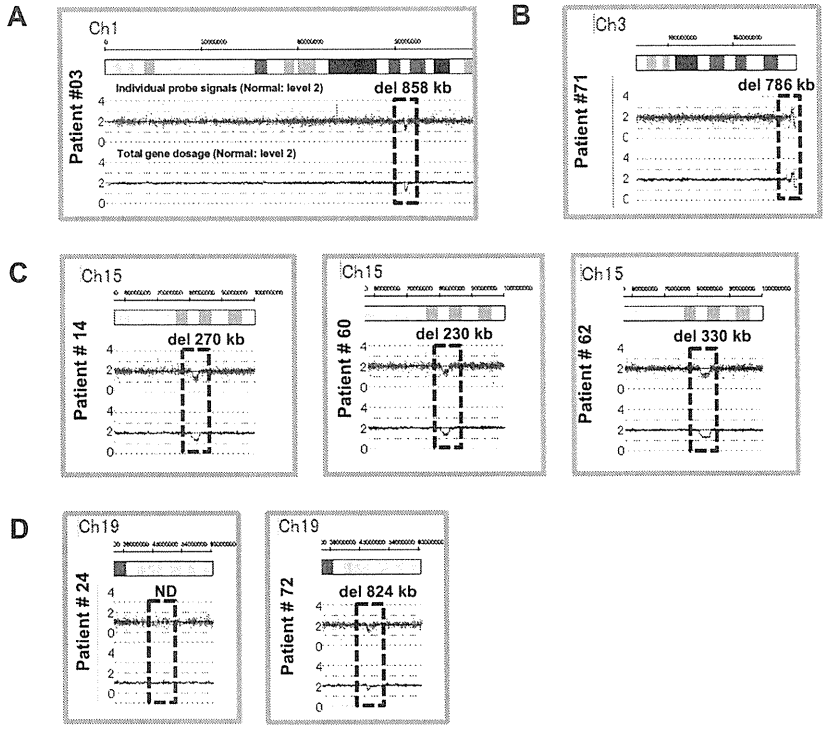

Figure 3. Results of SNP genomic microarray (SNP-chip) analysis. Genomic DNA of 27 Japanese DBA patients with unknown mutations was examined using a SNP array. Six patients had large deletions in their chromosome (ch), which included one DBA-responsible gene. Patient 3 has a large deletion in ch1 (A), 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

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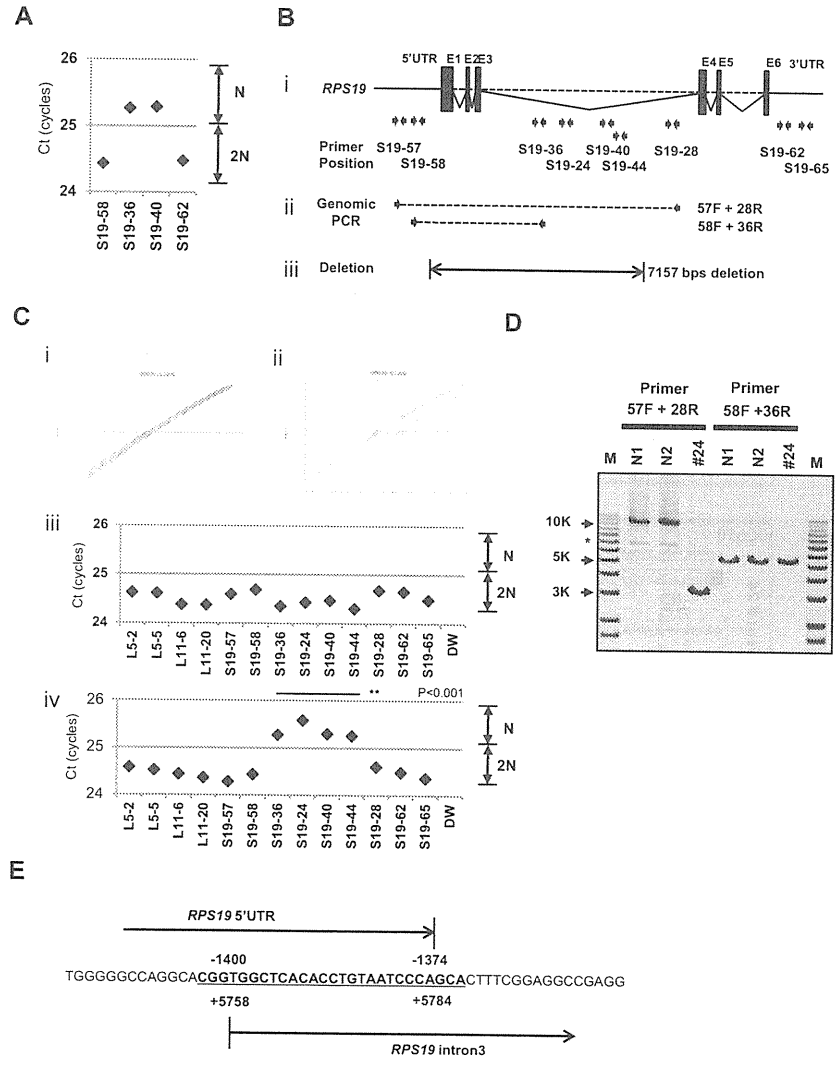

Figure 4. Result of s-q-PCR gene copy number assay for patient 24. (A) Results of s-q-PCR gene copy number assay for *RPS19* with 4 primer sets. (Bi) The *RPS19* gene copy number was analyzed with 9 specific primer sets for *RPS19* that span from the 5′UTR to the 3′UTR. (ii) Primer positions of genomic 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

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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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Brief report

Identification of *TRIB1* R107L gain-of-function mutation in human acute megakaryocytic leukemia

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Trib1 has been identified as a myeloid oncogene in a murine leukemia model. Here we identified a _TRIB1_ somatic mutation in a human case of Down syndrome–related acute megakaryocytic leukemia. The mutation was observed at well-conserved arginine 107 residue in the pseudokinase domain. This R107L mutation remained in leukocytes of the remission stage in which _GATA1_ mutation disappeared, suggesting the _TRIB1_ mutation is an earlier genetic event in leukemogenesis. The bone marrow transfer experiment showed that acute myeloid leukemia development was accelerated by transducing murine bone marrow cells with the R107L mutant in which enhancement of ERK phosphorylation and C/EBPα degradation by Trib1 expression was even greater than in those expressing wild-type. These results suggest that TRIB1 may be a novel important oncogene for Down syndrome–related acute megakaryocytic leukemia. (_Blood_. 2012; 119(11):2608-2611)

Introduction

The Down syndrome (DS) patients are predisposed to developing myeloid leukemia, and those patients frequently exhibit *GATA1* mutations.[1] However, it is proposed that the *GATA1* mutation is important for transient leukemia in DS but not sufficient for full-blown leukemia, suggesting that additional genetic alterations are needed.[1] Therefore, it is important to search the subsequent genetic changes for DS-related leukemia (ML-DS) to predict malignant transformation and prognosis of the patients.

Trib1 has been identified as a myeloid oncogene that cooperates with *Hoxa9* and *Meis1* in murine acute myeloid leukemia (AML).[2] As a member of the tribbles family of proteins, TRIB1 interacts with MEK1 and enhances ERK phosphorylation.[2,3] Moreover, TRIB1 promotes degradation of C/EBP family transcription factors, including C/EBPα, an important tumor suppressor for AML, and we observed that degradation of C/EBPα by Trib1 is mediated by its interaction with MEK1.[4] Thus, TRIB1 plays an important role in the development of AML by modulating both the RAS/MAPK pathway and C/EBPα function together with Trib2 that has also been identified as a myeloid-transforming gene.[5] Potential involvement of *TRIB1* in human leukemia has been reported in cases of AML with 8q34 amplification in which both *c-MYC* and *TRIB1* are included in the amplicon.[6] The enhancing effect of TRIB1 on the MAPK signaling suggests that TRIB1 alterations may be related to AML cases, which do not show any mutations in the pathway members, such as FLT3, c-Kit, or Ras. In this report, we identified a novel somatic mutation of *TRIB1* in a case of human acute megakaryocytic leukemia developed in DS (DS-AMKL). Retrovirus-mediated gene transfer followed by bone marrow transfer indicated that the mutation enhanced leukemogenic activity and MAPK phosphorylation by TRIB1.

Methods

Patients

TRIB1 mutations have been investigated in 12 cases of transient leukemia (TL), 5 of DS-AMKL, and 4 cell lines of DS-AML. Peripheral blood leukocytes of TL and bone marrow cells of DS-AMKL were used as sources for the molecular analysis. This study was approved by the Ethics Committee of Hirosaki University Graduate School of Medicine, and all clinical samples were obtained with informed consent from the parents of all patients, in accordance with the Declaration of Helsinki.

Patient 84 showed trisomy 21 and extensive leukocytosis at birth. Hematologic findings revealed the white blood cell count to be 148 × 10^9/L, including 87% myeloblasts, a hemoglobin level of 19.4 g/dL, and a platelet count of 259 × 10^9/L. Patent ductus arteriosus and atrial septal defect have been pointed out. Based on the hematologic data and the chromosomal abnormality, the patient was diagnosed as DS-related TL. The hematologic abnormality was then improved, but 8 months later 3% of 6.9 × 10^9/L white blood cells became myeloblasts (Figure 1A). A karyotype analysis of bone marrow cells revealed 48, XY,+8,+21 in 3 of 20 cells. In addition, *GATA1* mutation was detected at nt 113 from A to G, resulting in loss of the first methionine.[7] He was diagnosed as AMKL at this time, and his disease was in remission by subsequent chemotherapy.

PCR and sequencing

The entire coding region of human *TRIB1* cDNA of patients' samples was amplified using Taq polymerase (Promega) and specific primer pairs (the sequences of the primers are available on request). The genomic DNA samples of patient 84 were also analyzed. The sequence analysis of *GATA1* was performed as described previously.[7] After checking the PCR products by agarose gel electrophoresis, the products were purified and directly sequenced.

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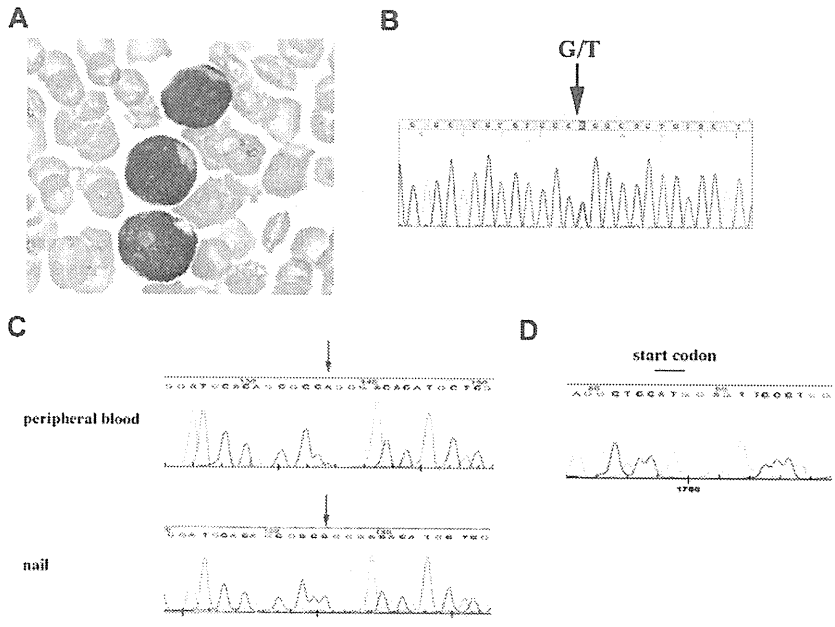

Figure 1. *TRIB1* R107L mutation identified in DS-related leukemias. (A) Giemsa staining of the case 84 peripheral blood smear diagnosed as AMKL. The image was acquired using a BX40 microscope equipped with a 100×/1.30 NA oil objective (Olympus) and a C-4040 digital camera (Olympus). (B) Fluorescent dye sequencing chromatographs of *TRIB1* genotyping by direct sequencing of the case 84 using a cDNA sample as a template. The vertical arrow indicates mixed G and T signals at codon 107. (C) Fluorescent dye sequencing chromatographs of *TRIB1* of peripheral blood leukocytes (top) or nail (bottom) in the same case at the complete remission stage. The red arrows indicate that the mutation remains in leukocytes but not in nail. The reverse strand sequences are shown. (D) *GATA1* sequence. The start codon that was mutated in AMKL[7] is normal in the peripheral blood leukocytes at the remission stage.

Retroviral infection of murine bone marrow cells and bone marrow transfer

Bone marrow cells were prepared from 8-week-old female C57Bl/6J mice 5 days after injection of 150 mg/kg body weight of 5-fluorouracil (Kyowa Hakko Kogyo). Retroviral infection of bone marrow cells and bone marrow transfer experiments were performed as described.[2] Transduction efficiencies evaluated by flow cytometric techniques were comparable between wild-type (WT; 5.3%) and R107L (3.4%). Animals were housed, observed daily, and handled in accordance with the guidelines of the animal care committee at Japanese Foundation for Cancer Research. All the diseased mice were subjected to autopsy and analyzed morphologically, and the blood was examined by flow cytometric techniques. The mice were diagnosed as positive for AML according to the classification of the Bethesda proposal.[8] The survival rate of each group was evaluated using the Kaplan-Meier method, and differences between survival curves were compared using the log-rank test.

Immunoblotting

Immunoblotting was performed using cell lysates in RIPA buffer as described.[4] Anti-p44/42 ERK (Cell Signaling Technologies), anti–phospho-p44/42 ERK (Cell Signaling Technologies), anti-C/EBPα (Santa Cruz Biotechnology), anti-FLAG (Sigma-Aldrich), and anti-GAPDH (Hy Test Ltd) antibodies were used.

Results and discussion

The important role of TRIB1 on the MAPK signaling suggests that *TRIB1* alterations may occur in some AML cases, which do not show overlapping mutations in the pathway members, such as FLT3, KIT, or RAS. Therefore, we tried to search mutations of *TRIB1* in cases of ML-DS and TL in which such mutations are infrequent.[9] In a case of DS-AMKL (case 84), a nucleotide change from guanine to thymine has been identified at 902 that results in amino acid alteration from arginine 107 (R107) to leucine (Figure 1B). The sequence changes were confirmed by subcloning the PCR product into the TA-type plasmid vector (data not shown). The nucleotide change was not observed in the DNA sample derived from the nail of the same patient at all (Figure 1C), indicating that this change is a somatic mutation. Interestingly, the mutation was retained in the peripheral blood sample in the complete remission stage in which the GATA1 mutation completely disappeared (Figure 1C-D). These results indicate that the *TRIB1* mutation precedes the onset of TL and the *GATA1* mutation, and suggest that *TRIB1* mutation occurred at the hematopoietic stem cell level and that the clone retaining the *TRIB1* mutation survived after chemotherapy. In case 84, there was no mutation for *FLT3* exons 14, 15, and 20, *PTPN11* exons 3 and 13, *KRAS* exons 2, 3, and 5, and *KIT* exons 8, 11, and 17 by the high-resolution melt analysis (data not shown).

An additional mutation was found in a case of TL (case 109) at the nucleotides 805 and 806 from GC to AT, which results in amino acid conversion from alanine (A75) to isoleucine (supplemental Figure 1, available on the *Blood* Web site; see the Supplemental Materials link at the top of the online article). *TRIB1* expression in DS-related and DS-unrelated leukemias was examined by real-time quantitative RT-PCR (supplemental Figure 2).

R107 is located within a psuedokinase domain of TRIB1 that is considered as a functionally core domain of TRIB family proteins.[10] Sequence comparison among 3 TRIB family proteins as well as tribbles homologs in other organisms revealed that the R107 is well conserved in mammalian TRIB1 and TRIB2,[10] suggesting that this arginine residue is evolutionary conserved and may be related to an important function. On the other hand, A75 is located outside of the pseudokinase domain, not conserved between human and mouse, or other tribbles homologs. Moreover, the N-terminal domain containing A75 is dispensable for the leukemogenic activity of Trib1.[4] Therefore, we tried to investigate whether the R107L mutation could affect the leukemogenic activity of *TRIB1*.

R107L was introduced into the murine *Trib1* cDNA by site-directed mutagenesis. Both WT and R107L cDNAs were subcloned into the pMYs-IRES-GFP retroviral vector and were used for retrovirus-mediated gene transfer followed by bone marrow transfer according to the method previously described.[1] All the mice

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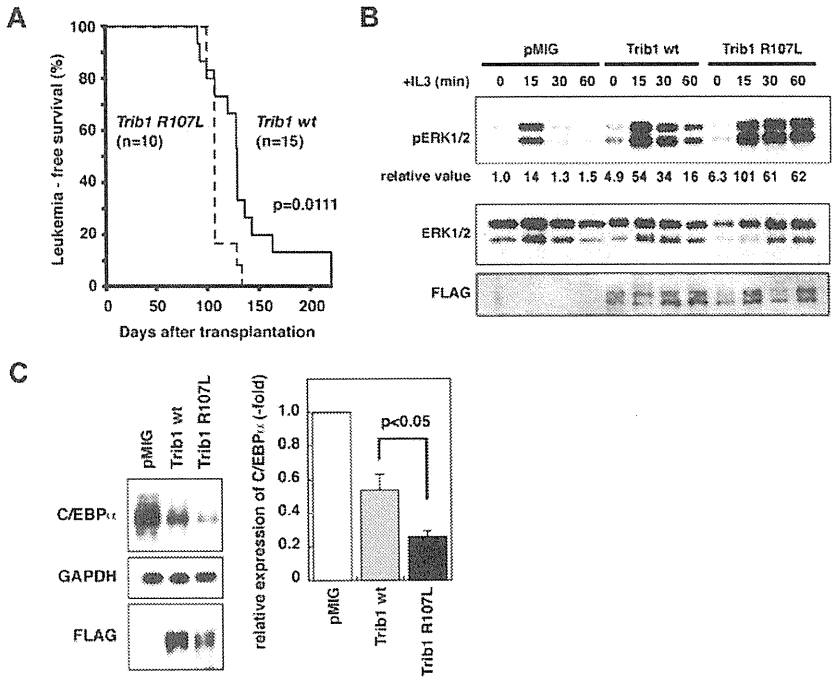

Figure 2. AML development by bone marrow transfer using *Trib1* WT and R107L. (A) Kaplan-Meier survival curves are shown. The *P* value was calculated with the log-rank test. (B) Immunoblot analysis of *Trib1* WT AML (Mac-1 56.2%, Gr-1 52.5%, CD34^{lo}, c-kit^{-}, Sca-1^{-}) and R107L AML (Mac-1 41.4%, Gr-1 25.2%, Cd34^{lo}, c-kit^{lo}, Sca-1^{-}) derived from bone marrow of recipient mice (WT #T73 and R107L #T151 in supplemental Table 1). Enhancement of ERK phosphorylation is more significant in R107L. Relative values of ERK phosphorylation were calculated by densitometric analysis. (C) Immunoblot analysis for C/EBPα of the same AML samples as in panel B. Relative expression level of C/EBPα is quantitated (right).

transplanted with bone marrow cells expressing WT (n = 15) or R107L (n = 12) developed AML (Figure 2A). The mean survival time was shorter in the recipients with R107L-expressing bone marrow cells (110 days) than those with WT (136 days; Figure 2A). The difference was significant (P = .0111, log-rank test). The result indicates that the R107L mutation enhances the leukemogenic activity of TRIB1. These results also suggest that *TRIB1* mutation might cooperate with *GATA1* mutation in the genesis of DS-AMKL, and that trisomy 21, *TRIB1*, and *GATA1* mutations occurred consecutively, which contributed to the multistep leukemogenic process.

We have shown that TRIB1 interacts with MEK1 and enhances phosphorylation of ERK.[2] The R107L mutant enhanced ERK phosphorylation more extensively than WT (Figure 2B) in AML cells derived from bone marrow of recipient mice, and more significant degradation of C/EBPα was induced by the R107L mutant (Figure 2C). These findings might be correlated to the enhanced leukemogenic activity of the mutant. Both R107L and WT proteins could interact with MEK1, having the binding motif in their C-termini. The residue 107 is located at subdomain II of the pseudokinase domain.[11] The mutation may affect conformation of the domain and may promote the MEK1 function on ERK, although additional studies are required to address the possibility. A recent study demonstrates that Trib1 and Trib2 failed to show ERK phosphorylation in 32D cells.[12] The different response to Trib1 between primary leukemic cells and the cell line might depend on the cellular context and/or combination of additional mutations. The AML phenotypes were somewhat varied in each case and Mac-1–positive/Gr-1–negative AMLs were more remarkable in WT than in R107L, although the difference was not statistically significant (supplemental Figures 3-4; supplemental Table 1). The current study underscores the role of TRIB1 in human leukemogenesis and the significance of the R107L mutation in its function. Further sequence analysis of tribbles family genes in a larger cohort will emphasize the importance of R107L and/or additional mutations of *TRIB1* in leukemic patients.

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Authorship

Contribution: T.Y., E.I., Y.H., and T.N. designed and performed the research and wrote the manuscript; T. Toki, Y.A., R.K., and M.-j.P. performed the research; and Y.K., T. Takahara, and Y.Y. contributed to the bone marrow transplantation analysis.

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Cord Blood Transplantation from Unrelated Donors for Children with Acute Lymphoblastic Leukemia in Japan: The Impact of Methotrexate on Clinical Outcomes

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Cord blood transplantation (CBT) from an unrelated donor is recognized as one of the major treatment modalities in allogeneic stem cell transplantation (SCT) for children with hematologic malignancies. We analyzed the clinical outcomes of CBT for children with acute lymphoblastic leukemia (ALL) in Japan and identified the risk factors for the transplant outcomes. From 1997 to 2006, 332 children with ALL underwent CBT from unrelated donors, 270 of which had no prior transplant. Their disease statuses at transplant were first complete remission (CR) (n = 120), second CR (n = 71), and more advanced stages (n = 75). As preconditioning for SCT, total body irradiation (TBI) was given to 194 patients and, for the prophylaxis of graft-versus-host disease (GVHD), methotrexate (MTX) was given to 159 patients. The cumulative incidents of neutrophil and platelet recovery (>20 K) were 88.5% and 78.4%, respectively. The incidents of grade II-IV, III-IV acute GVHD (aGVHD), and chronic GVHD (cGVHD) were 45.6%, 20.4%, and 19.2%, respectively, and treatment-related mortality was 22.6%. The 5-year event-free survival (EFS) and overall survival (OS) at CR1, CR2, and advanced status were 47.4%, 45.5%, 15.0%, and 63.7%, 59.7%, and 20.7%, respectively. Multivariate analysis revealed that MTX with calcineurin inhibitor (CNI) was associated with decreased incidence of grade II-IV GVHD (CNI alone: hazard ratio [HR] = 1.74, 95% confidence interval [CI] = 1.06-2.83, $P = .027$; CNI + prednisolone (PSL), HR = 1.61, 95% CI = 1.03-2.50, $P = .036$), III-IV aGVHD (CNI alone: HR = 3.02, 95% CI = 1.55-5.91, $P = 0.001$; CNI + PSL, HR = 1.89, 95% CI = 0.93-3.83, $P = .078$), or cGVHD (CNI alone: HR = 1.78, 95% CI = 0.83-3.82, $P = .143$; CNI + PSL, HR = 2.44, 95% CI = 1.24-4.82, $P = .01$), compared with CNI alone or CNI + PSL. At an advanced stage of disease, GVHD prophylaxis with MTX + CNI is associated with improved OS compared with CNI alone (CNI alone: HR = 3.20, 95% CI = 1.43-7.15, $P = .005$; CNI + PSL, HR = 1.47, CI = 0.67-3.20, $P = .332$). Our retrospective study showed that CBT for children with ALL is feasible and GVHD prophylaxis with MTX + CNI is associated with significant favorable outcomes in prevention of aGVHD and cGVHD as well as survival advantage in advanced cases.

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KEY WORDS: Cord blood transplantation, Acute lymphoblastic leukemia, HLA, Methotrexate

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INTRODUCTION

Multiagent chemotherapy for children with acute lymphoblastic leukemia (ALL) has achieved excellent clinical outcomes in recent years [1,2]. However, those patients who relapsed during or after chemotherapy or those with very high-risk features, such as Philadelphia chromosome-positive ALL (Ph+ALL) or infant ALL with mixed lineage leukemia (MLL) gene rearrangement, are proposed as candidates for allogeneic stem cell transplantation (SCT) [3-6] at their first remission. Patients and donors are required to be compatible in terms of human leukocyte antigen (HLA) for better transplant outcome and, if they lack an HLA-identical related donor, they have options of alternative donors, such as bone marrow transplantation (BMT), peripheral blood stem cell transplantation (PBSCT), cord blood transplantation (CBT) from an unrelated donor, or transplantation from an HLA-haploidentical family donor [7-9]. Out of these four treatment modalities, CBT has advantages such as immediate availability of a CB unit for an urgent transplant, lower risks of severe acute and chronic graft-versus-host disease (aGVHD, cGVHD), and a less stringent requirement of HLA compatibility than unrelated or haploidentical BMT. In Japan, the Japan Cord Blood Bank Network (JCBBN) was established in 1999, and 11 local cord blood banks are affiliated to JCBBN where more than 7000 CBT were performed by the end of 2010. Here, we report the clinical outcomes and risk factors of children with ALL who underwent CBT in Japan.

PATIENTS AND METHODS

Patient and Donor Characteristics

From 1997 to 2006, 332 unrelated CBT were performed for children with ALL and 270 transplantations were undertaken as the first SCT in Japan. Because the overall survival (OS) of patients who underwent transplantation as the first SCT was significantly better than that of those with prior SCT (50.3% vs 12.7%, $P < .001$), we restricted this analysis to only patients with no prior SCT in order to interpret the exact risk factors of CBT. The patient and donor characteristics are shown in Table 1. Patients transplanted at first complete remission (CR1) (n = 120) include 41 infant ALL and 17 patients with Ph +ALL.

The HLA typing of cord blood units was performed in each CB bank by low-resolution molecular typing of HLA-A and B, combined with high-resolution molecular typing of DRB1. The high-resolution molecular typing for 3 loci of HLA-A, B, and DRB1 was performed in 187 patients.

In JCBBN, CB units of 0 to 2 HLA antigen mismatches with the patient were allowed for transplantation, and the minimum number of nucleated cells recommended for transplantation was 2×10^7/kg of patient body weight at cryopreservation.

Transplantation

All CB units were provided from the 11 local CB banks affiliated to JCBBN, and all transplant institutions were required to meet the minimum requirements of JCBBN in terms of experience of allogeneic SCT. The numbers of transplanted cells, preconditioning, as well as GVHD prophylaxis are shown in Table 1. Supportive care after transplantation, such as gut decontamination, empirical administration of antibiotics, prophylaxis or treatment of cytomegalovirus (CMV) infection, was performed according to each institutional protocol. Grading of GVHD was performed according to the standard criteria [10].

Definition and Statistics

The median duration of follow-up was 438 days (range: 10-3293 days). In this study, rates of neutrophil and platelet engraftment, incidents of aGVHD and cGVHD, leukemic relapse, nonrelapse mortality (NRM), and probabilities of event-free survival (EFS) and OS were analyzed. The variables evaluated included recipient age, sex, sex mismatch, disease status at transplants (CR1/CR2 vs advanced disease), ABO compatibility, HLA matching by low- and high-resolution typing, number of nucleated cells, colony-forming unit-granulocyte-macrophage (CFU-GM) and CD34-positive cells of the cord blood units at cryopreservation conditioning regimens with total body irradiation (TBI), administration of granulocyte-colony stimulating factor (G-CSF), GVHD prophylaxis (calcineurin inhibitor [CNI] alone, CNI + methotrexate [MTX] versus CNI + prednisolone [PSL]), mixed lineage leukemia (MLL) gene rearrangement, t(4;11), and transplantation year. Because the information of high-resolution DNA typing was only available for a limited number of patients, it was not included in the multivariate analysis. The day of neutrophil engraftment was defined as the first day of 3 consecutive days with absolute neutrophil count (ANC) $\geq 500/mm^3$, and that of platelet engraftment was the first day of platelet count over $20,000/mm^3$ without transfusion. The treatment-related mortality was defined as all causes of nonleukemic deaths after transplantation. The EFS was defined as patients who are alive in CR with engraftment. The probabilities of OS and EFS were calculated by the method of Kaplan and Meier. The log-rank test was used for group comparisons. Time-to-event outcomes for neutrophil and platelet engraftment, treatment-related mortality, relapse,

Table 1. Patient and Donor Characteristics

Total Number of Patients			270
Age (year)	median (range)		5 (0-15)
Body weight (kg)	median (range)		18 (4-60)
Sex	male/female		156/114
Duration from diagnosis to transplantation (days)	median (range)		249 (94-3670)
Disease status at transplantation (patients)	CR1		120
	CR2		71
	advanced		75
	unknown		4
Cytogenetics (patients)	Philadelphia chromosome		31
	MLL gene rearrangement		73
	t(4;11)		40
Preparative regimen (patients)	TBI regimen		194
		TBI + CY + VP16	68
		TBI + CY ± others	67
		TBI + L-PAM ± others	56
		Others	3
	non-TBI regimen		76
		BU + CY ± others	55
		Others	21
G-CSF (patients)	+		249
	−		21
GVHD prophylaxis (pts)	CNI only	cyclosporine	29
		tacrolimus	12
	CNI + MTX	cyclosporine + MTX	83
		tacrolimus + MTX	66
	CNI + PSL	cyclosporine + PSL	36
		tacrolimus + PSL	11
	ATG + CNI ± MTX		7
	others		15
	none		11
Number of cells at cryopreservation, median (range)	Nucleated cell ($\times 10^7$/kg)	(n = 270)	5.00 (1.35-24.91)
	CFU-GM ($\times 10^3$/kg)	(n = 258)	34 (0.87-473.2)
	CD34 ($\times 10^5$/kg)	(n = 207)	1.49 (0.17-15.02)
Blood type of donor and recipient (pts)	match		89
	minor		77
	major		103
	unknown		1
Sex of donor and recipient (pts)	M to M		78
	F to F		64
	M to F		50
	F to M		78
HLA disparity in low resolution (patients)	No. of disparities	GVHD direction	Rejection direction
	0	54	57
	1	168	167
	2	47	45
	unknown	1	1
HLA disparity in high resolution (patients)	No. of disparities	GVHD direction	Rejection direction
	0	21	23
	1	56	58
	2	77	72
	3	28	29
	4	4	4
	5	1	1
	unknown	83	83

G-CSF indicates granulocyte-colony stimulating factor; GVHD, graft-versus-host disease; TBI, total body irradiation; CNI, calcineurin inhibitor; MTX, methotrexate; PSL, prednisolone; CY, cyclophosphamide.

and GVHD were estimated using cumulative incidence curves. The competing risk of engraftment is death before engraftment, that of GVHD is death without GVHD or relapse, and that of relapse is death without relapse. The Cox proportional-hazards regression model was used for multivariate analysis of clinical variables. P values $<.05$ were considered statistically significant. Risk factors with a P value $<.1$ in each univariate analysis were included in the multivariate analysis. STATA version 10 (Stata Corporation, College Station, TX) and NCSS 2004 (Number Cruncher Statistical Systems, Kaysville, UT) were used for the statistical analysis of data.

RESULTS

Neutrophil Engraftment

Neutrophil engraftment was obtained in 239 patients. The probability of neutrophil engraftment

was 88.5% (95% confidence interval [CI], 84.8%-92.4%) by day 90, and the median number of days to reach ANC over 500/mm^3 was 22. In univariate analysis, younger versus older than 1 year old (92.3% vs 87.6%, P = .001), higher versus lower than 3 × 10^7/kg of nucleated cells (89.5% vs 84.6%, P = .003), higher versus lower than the median number of CFU-GM (90.8% vs 86.8%, P < .001), higher versus lower than the median number of CD34$^+$ cells (1.5 × 10^5/kg, 89.7% vs 85.1%, P < .001), 0-1 versus 2 Ag HLA mismatches in either GVHD (89.2% vs 85.1%, P = .008) or rejection (90.2% vs 80.0%, P = .004) direction, allelic 0-1 versus 2 or more HLA mismatches in either GVHD (92.2% vs 87.3%, P = .009) or rejection (92.6% vs 86.8%, P = .006) direction by high-resolution typing, CR1 or CR2 versus advanced status at transplantation (89.5% vs 85.3%, P = .022), and presence versus absence of G-CSF (91.2% vs 57.1%, P < .001) were significantly associated with higher neutrophil engraftment rate. The presence or absence of MTX did not affect the neutrophil engraftment (data not shown). In multivariate analysis, favorable predictive factors of neutrophil engraftment were higher number of CD34$^+$ cells, administration of G-CSF, and HLA disparity of 0-1 antigen for rejection direction (Table 2).

Platelet Engraftment

Platelet engraftment over 20,000/mm^3 was obtained in 202 patients, and the probability of platelet engraftment by day 180 was 78.4%. In univariate analysis, younger versus older than 1 year old (82.8% vs 77.1%, P = .004), higher versus lower than the median number of nucleated cells (78.8% vs 76.6%, P = .008), higher versus lower than the median number of CFU-GM (82.8% vs 73.7%, P = .004), higher versus lower than the median number of CD34$^+$ cells (84.9% vs 71.8%, P < .001), disease status of CR1 or CR2 versus advanced (83.8% vs 63.3%, P < .001), and presence versus absence of G-CSF (80.1% vs 57.8%, P = .017) were significantly associated with higher platelet engraftment rate. Multivariate analysis revealed that a higher number of CD34$^+$ cells and CR1 or CR2 at transplantation were favorable prognostic factors for platelet engraftment (Table 2).

GVHD

The cumulative incidents of grade II-IV and III-IV aGVHD were 45.6% (95% CI, 40.0%-51.9%) and 20.4% (95% CI, 16.1%-25.8%), respectively. In univariate analysis, HLA-mismatched donor versus matched donor in GVHD direction by low resolution (49.3% vs 31.5%, P = .023) and high resolution (51.2% vs 14.3%, P = .003), and presence versus absence of TBI (51.6% vs 30.3%, P = .003) were significantly associated with the development of grade II-IV aGVHD, and MTX + CNI showed a trend of impact on the development of grade II-IV aGVHD (40.3% in MTX + CNI, 53.7% in CNI alone, and 63.8% in CNI + PSL, P = .096). GVHD prophylaxis with MTX + CNI was the only significant predictive factor for decreased incidence of grade III-IV GVHD (14.1% in MTX + CNI, 27.7% in CNI + PSL and 36.7% in CNI alone, P = .011) (Figure 1). Multivariate analysis revealed that TBI was significantly associated with increased incidence of grade II-IV aGVHD, and GVHD prophylaxis with MTX + CNI was significantly associated with decreased incidence of grade II-IV and III-IV aGVHD (Table 2).

The cumulative incidence of the development of cGVHD was 19.2% (95% CI, 15.0%-24.6%), and the incidence of cGVHD was significantly reduced in HLA-matched donor in low resolution for rejection direction compared with that in the GVHD direction (12.2% vs 22.6%, P = .002), as well as GVHD prophylaxis with MTX + CNI compared with that with CNI alone or CNI + PSL (16.1% vs 22.5%, or 29.3%, respectively, P = .03) (Figure 1). In multivariate analysis, HLA mismatch for rejection direction in low resolution and GVHD prophylaxis with CNI + PSL were the significant risk factors for the development of cGVHD (Table 2). In our study population, only 7 patients were given anti-T cell globulin (ATG) for GVHD prophylaxis. The cumulative incidence of grade II-IV aGVHD and cGVHD in this population was 14.7%, respectively, and 5 patients died of either relapse or transplantation-related complications.

Transplant-Related Mortality (TRM)

The cumulative incidence of TRM after CBT was 22.6% (95% CI, 17.7%-27.8%). Univariate analysis showed that HLA mismatch of 2 or more loci versus 0-1 in high-resolution typing for either GVHD direction (23.2% vs 10.4%, P = .03) or rejection direction (23.1% vs 11.2%, P = .03), advanced disease status versus CR1 or CR2 (35.3% versus 17.4%, P < .001), and GVHD prophylaxis other than MTX + CNI (15.1% in MTX + CNI, 29.3% in CNI alone, and 31.4% in CNI + PSL, P = .01) were significantly associated with a higher incidence of TRM. Multivariate analysis revealed that advanced disease status at transplantation was a risk factor for TRM (Table 2).

Leukemic Relapse

Eighty-six patients relapsed between 8 and 976 days (median 182) after CBT. The cumulative incidence of leukemic relapse at 3 years was 35.2% (95% CI, 29.8%-42.1%). Advanced disease versus CR1 or CR2 (48.8% vs 30.6%, P < .001) and presence

Table 2. Multivariate Analysis of Risk Factors for Transplantation Outcomes

	Variable		Hazard Ratio	*P* Value	95% CI
Neutrophil engraftment	CD34 ($\times 10^5$/kg)	<1.5	1		
		≥1.5	1.7	.001	1.26-2.28
	G-CSF	no	1		
		yes	3.06	.001	1.60-5.83
	HLA disparity in low resolution (rejection direction)	0-1	1		
		2	0.62	.024	0.41-0.94
Platelet engraftment (≥20,000/mm^3)	CD34 ($\times 10^5$/kg)	<1.5	1		
		≥1.5	1.9	.001	1.35-2.66
	Disease status	CR1, CR2	1		
		advanced	0.58	.008	0.39-0.87
Acute GVHD (≥II)	TBI	no	1		
		Yes	1.859	.015	1.13-3.06
	GVHD prophyalxis	CNI + MTX	1		
		CNI only	1.74	.027	1.06-2.83
		CNI + PSL	1.61	.036	1.03-2.50
Acute GVHD (≥III)	GVHD prophylaxis	CNI + MTX	1		
		CNI only	3.02	.001	1.55-5.91
		CNI + PSL	1.89	.078	0.93-3.83
Chronic GVHD	HLA disparity in low resolution (GVHD direction)	0	1		
		1,2	2.73	.055	0.98-7.61
	GVHD prophylaxis	CNI + MTX	1	.029	
		CNI only	1.777	.143	0.83-3.82
		CNI + PSL	2.44	.01	1.24-4.82
Treatment-related mortality	Disease status	CR1, CR2	1		
		advanced	2.56	.005	1.33-4.92
Relapse	Disease status	CR1, CR2	1		
		advanced	3.16	<.001	2.04-4.89
	t(4;11)	no	1		
		yes	1.93	.014	1.14-3.26
Overall survival	Disease status	CR1, CR2	1		
		advanced	3.62	<.001	2.44-5.8
Event-free survival	Disease status	CR1, CR2	1		
		advanced	2.54	<.001	1.83-3.51

CI indicates confidence interval; G-CSF indicates granulocyte-colony stimulating factor; GVHD, graft-versus-host disease; TBI, total body irradiation; CNI, calcineurin inhibitor; MTX, methotrexate PSL, prednisolone.

versus absence of t(4;11) chromosomal abnormality (48.3% vs 33.1%, $P = .044$) were significantly associated with leukemic relapse in univariate analysis. Both of these factors were also significant in multivariate analyses (Table 2).

OS

One hundred fifty-two patients were alive after CBT, and their median number of days of survival was 961 (91-3293). The cause of death in 118 patients included relapse or progressive disease (n = 50), TRM (n = 66), and unknown reason (n = 2). The probability of projected 5-year OS for all patients was 50.3% (95% CI, 43.4%-56.8%), and it was 63.7% in CR1, 59.7% in CR2, and 20.7% at more advanced disease status (Figure 2). Univariate analysis revealed that HLA mismatch of ≥2 versus 0 or 1 for rejection direction (50.9% vs 66.0%, $P = .017$) in high-resolution typing, advanced disease versus CR1 or CR2 (20.7% vs 62.1%, $P < .001$), and GVHD prophylaxis of other than MTX + CNI (56.8% in MTX + CNI, 43.3% in CNI alone, and 40.6% in CNI + PSL, $P = .049$) were significantly associated with OS (Figure 3). In multivariate analysis, advanced disease status at transplantation was the only risk factor for OS. When multivariate analysis was restricted to the patients with advanced diseases, OS was significantly superior for patients with GVHD prophylaxis of MTX + CNI than CNI alone (CNI alone: HR = 3.20, 95% CI = 1.43-7.15, $P = .005$; CNI + PSL, HR = 1.47, CI = 0.67-3.20, $P = .332$).

EFS

The probability of projected 5-year EFS for all patients was 38.1% (95% CI, 31.8%-44.4%), and it was 47.4% in CR1, 45.5% in CR2, and 15.0% at more advanced disease status. Univariate analysis revealed that HLA mismatch of 1 or more versus 0 with high-resolution typing in either GVHD direction (33.9% vs 51.3%, $P = .047$) or rejection direction (31.5% vs 52.9%, $P = .010$), advanced disease status versus CR1 or CR2 (15.0% vs 46.6%, $P < .001$), and absence versus presence of G-CSF (22.0% vs 39.3%, $P = .028$) were significantly associated with EFS. The EFS rates of patients according to the HLA disparity in high-resolution typing in GVHD direction were 61.9% in 0 of 6 (n = 21), 47.0% in 1 of 6 (n = 56), 36.4% in 2 of 6 (n = 77), and 28.4% in 3 of 6 (n = 28) ($P = .127$). Although this was not significant, the more the HLA disparity increased, the lower the EFS became. In multivariate analysis, advanced disease

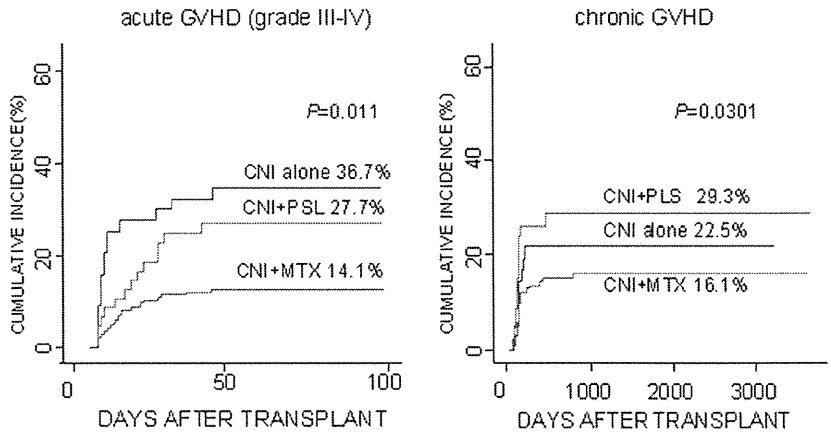

Figure 1. Cumulative incidence of aGVHD (grade III-IV) (left) and cGVHD (right). GVHD prophylaxis with CNI + MTX is associated with significantly lower incidence of aGVHD and cGVHD.

status at transplantation was significantly associated with lower EFS (Table 2).

DISCUSSION

The outcomes of CBT according to the disease status at transplantation in children with ALL were reported from a multicenter study of Eurocord. The disease-free survival (DFS) rates of those patients transplanted at complete remission and at more advanced stages were 36%-49% and 10%-18%, respectively [11-13]. In contrast to these multicenter studies, single or small numbers of institutions report better results. A study from Minnesota University reported that the EFS of children with ALL in standard and high-risk patients are 55% and 32%, respectively [14]. In the Cord Blood Transplantation (COBLT) study, the OS of children with ALL was around 60% in first and second remission [15], and a study in Denver [16] reported DFS of 62% including standard and high-risk patients. Our study is a retrospectively reviewed multicenter study with a large number of children with ALL, and the EFS or OS is comparable to that of these single-center studies.

The relevance of HLA disparity to clinical outcome in unrelated CBT has been reported by several investigators. In an International Bone Marrow Transplant Registry (IBMTR) study, the OS of serologically 6/6-matched CBT was significantly better than that of mismatched CBT, irrespective of the cell dose of the CB unit [17]. In Eurocord, the serologic disparity of HLA was reported to be important for engraftment and relapse but not for GVHD or survival, namely, serologic HLA mismatch reduced the relapse rate after transplantation. In those studies, HLA disparity in high resolution did not affect any clinical outcomes [18]. In our study, HLA disparity in low resolution affected the neutrophil engraftment and GVHD or cGVHD but not for relapse and survival. The OS according to the HLA disparity in high resolution gradually declined as the HLA disparity increased, even though this was not statistically significant in univariate analysis.

The different results regarding risk factors for relapse between our data and Eurocord may be explained by the difference of the patient population. Our study

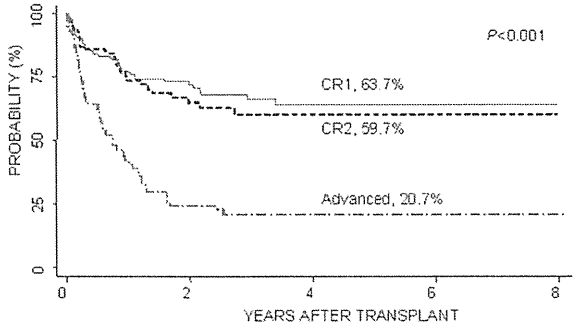

Figure 2. Probability of overall survival of patients according to disease status at transplantation. Patients with CR1 and CR2 are associated with significantly better overall survival compared with patients with advanced stage.

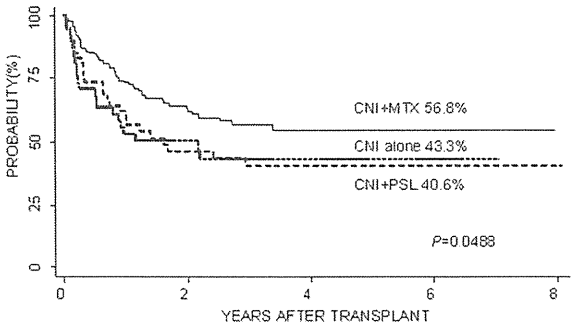

Figure 3. Probability of overall survival of patients according to GVHD prophylaxis. Patients with GVHD prophylaxis of CNI + MTX is associated with significantly better overall survival compared with patients with CNI alone or CNI + PSL.

was restricted to childhood ALL, whereas Eurocord included acute myelogenous leukemia (AML) patients [12,18], for whom a graft-versus-leukemia (GVL) effect could be more efficient than ALL patients after allogeneic SCT. Although the implications of the HLA disparity in high resolution for clinical outcome are still controversial, future study with a large number of patients could clarify the relevance of HLA disparity in high resolution on clinical outcomes.

GVHD prophylaxis after CBT is still controversial, and various methods of prophylaxis are applied in each institution or study group. In the early era of unrelated CBT, cyclosporine (CsA) and steroids with or without MTX were given as GVHD prophylaxis [19]. Subsequently, MTX was abandoned, and immunosuppression with CsA and steroids became popular in the United States and European countries. In their reports, the incidence of GVHD after CBT is 35% to 44% for grade II-IV aGVHD, 11% to 27% for grade III-IV aGVHD, and 9% to 33% for cGVHD [14,20-22], mostly by prophylaxis with CsA and steroids. GVHD prophylaxis with CNI alone after CBT was reportedly complicated with preengraftment immune reaction [23], but a Japanese retrospective study showed the superiority of GVHD prophylaxis with 2 agents compared with that of single agent in terms of DFS for patients with acute leukemia [24]. In this study, we found that the use of MTX showed favorable effects of significantly lower incidents of aGVHD and cGVHD, and in advanced cases, better OS was observed without affecting the engraftment or relapse. In Eurocord, an unfavorable effect of delayed myeloid engraftment by MTX was reported only in related CBT but not in unrelated CBT [25,26]. Another disadvantage of MTX reported by Eurocord was a higher relapse rate in unrelated CBT for children with ALL [12]. This unfavorable effect was not observed in our study, and this discrepancy could be explained by the different proportion of patients who were given ATG before SCT. In one Eurocord study for children with ALL, 88% of patients were given ATG [13], but only 7 of 270 patients (2.6%) were given ATG in our study. Because ATG reduces the incidence of aGVHD and cGVHD by purging T cells in vivo [27], GVHD prophylaxis including MTX with or without ATG needs to be analyzed in terms of transplantation outcomes including the GVL effect.

In Japan, Narimatsu [28] and Terakura [29] reported that MTX after CBT reduced the complications such as preengraftment immune reaction, engraftment syndrome, and aGVHD, as well as the incidence of treatment-related mortality and improved survival in adults. Takahashi also reported superior DFS after CBT with GVHD prophylaxis of MTX and CsA [30]. Neither of these studies found any unfavorable effects caused by MTX in unrelated CBT. In a Japanese pediatric study of CBT for AML, MTX contributed to lower TRM [31]. The critical role of MTX in unrelated CBT should be emphasized as a key drug in terms of prophylaxis for GVHD, although transplantation outcomes according to the dose and frequency of MTX administration was unable to be analyzed in this study. In our study, nobody was given mycophenolate mofetil (MMF), and the combination of CNI + MMF needs to be compared with CNI + MTX in the pediatric population.

In conclusion, CBT from an unrelated donor is feasible and effective as a treatment modality for children with ALL, and GVHD prophylaxis, which includes MTX, is critical to reduce the incidence of aGVHD and cGVHD without affecting engraftment, as well as to achieve better OS in advanced cases.

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