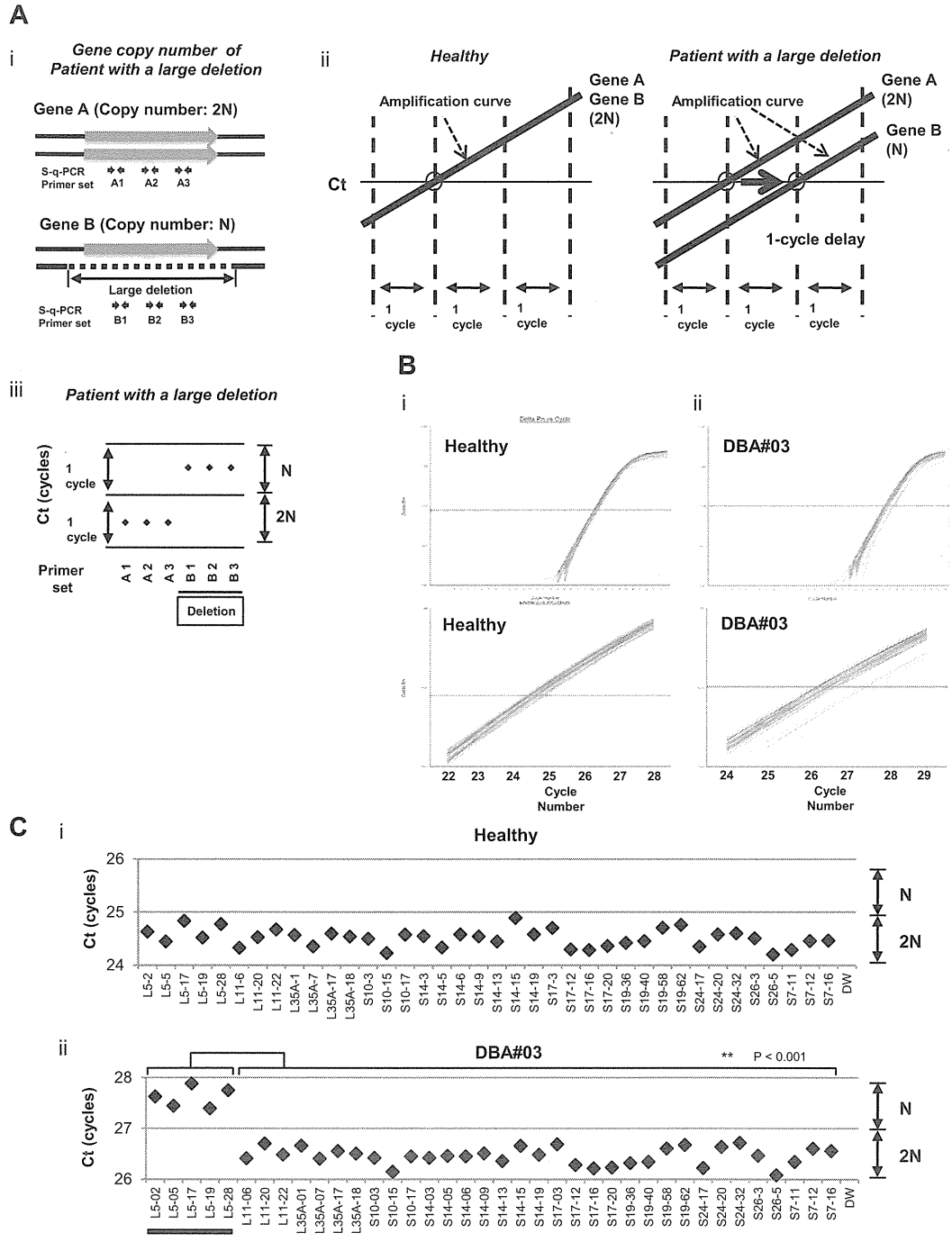


**Figure 1. s-q-PCR can determine a large gene deletion in DBA.** (A) Concept of the DBA s-q-PCR assay. The difference in gene copy number between a healthy sample and that with a large deletion is 2-fold (i). When all genomic s-q-PCR for genes of interest synchronously amplify DNA fragments, a 2-fold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

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

## Methods

### Patient samples

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

### DBA gene copy number assay by s-q-PCR

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

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

### Genomic PCR

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

### DNA sequencing analysis

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

### SNP array-based copy number analysis

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

## Results

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

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

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

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

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

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

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

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

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

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

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

**Table 4. Characteristics of DBA patients tested**

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

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

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

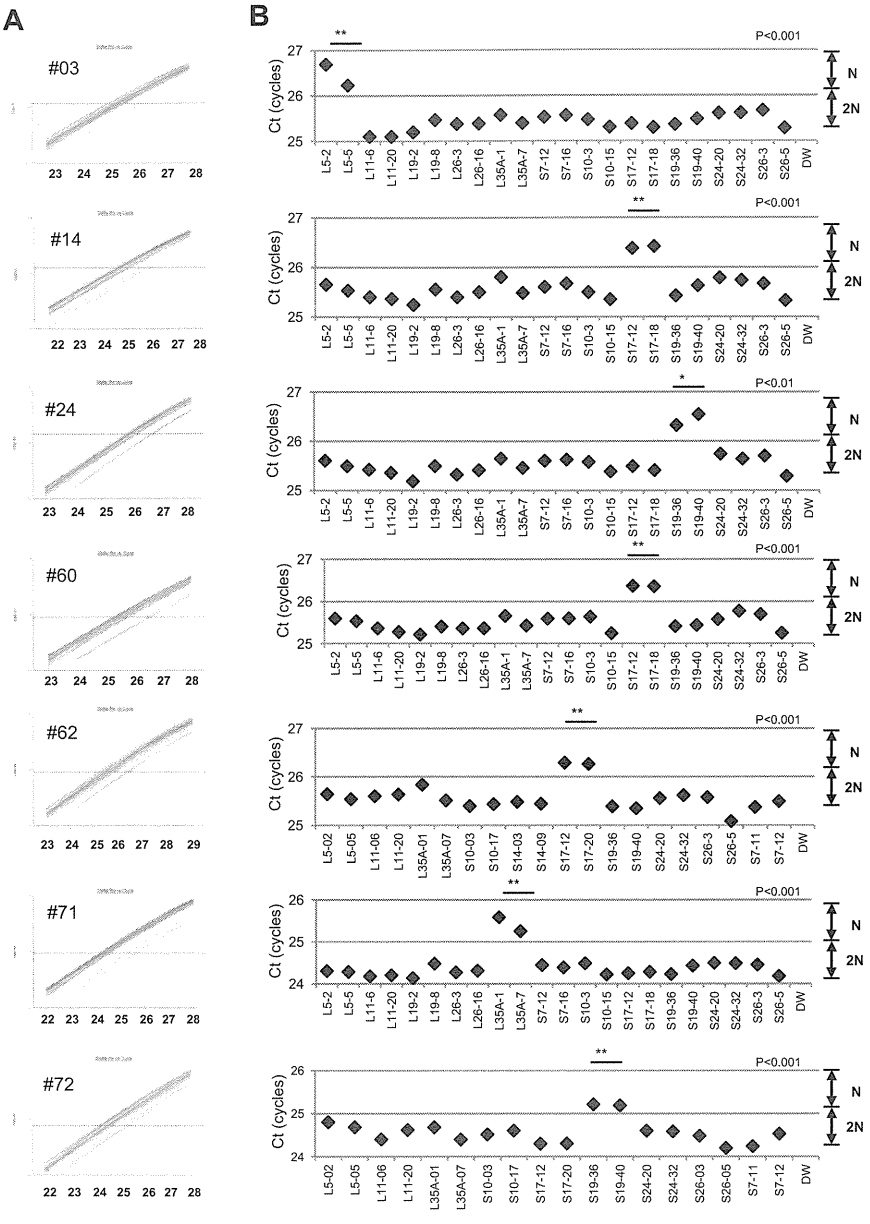


**Figure 2. Detection of 7 mutations with a large deletion in DBA patients.** Genomic DNA of 27 Japanese DBA patients with unknown mutations were subjected to the DBA gene copy number assay. (A) Amplification curve of s-q-PCR of a mutation with a large deletion. The deleted gene can be easily distinguished.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

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

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

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

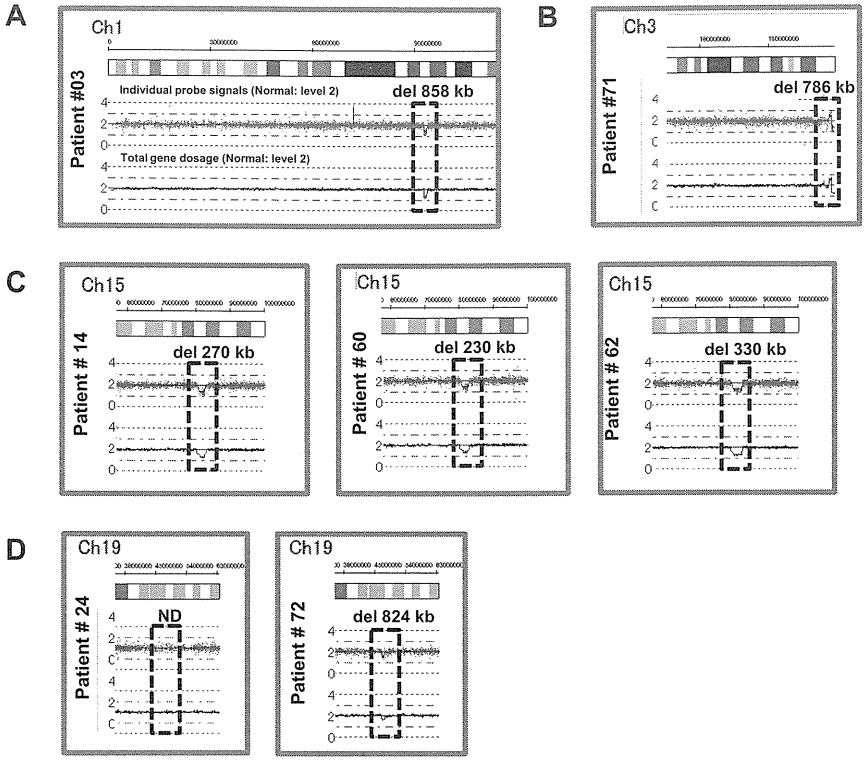


**Figure 3. Results of SNP genomic microarray (SNP-chip) analysis.** Genomic DNA of 27 Japanese DBA patients with unknown mutations was examined using a SNP array. Six patients had large deletions in their chromosome (ch), which included one DBA-responsible gene. Patient 3 has a large deletion in ch1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

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

### Genotype-phenotype analysis and DBA mutations in Japan

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

## Discussion

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

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

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

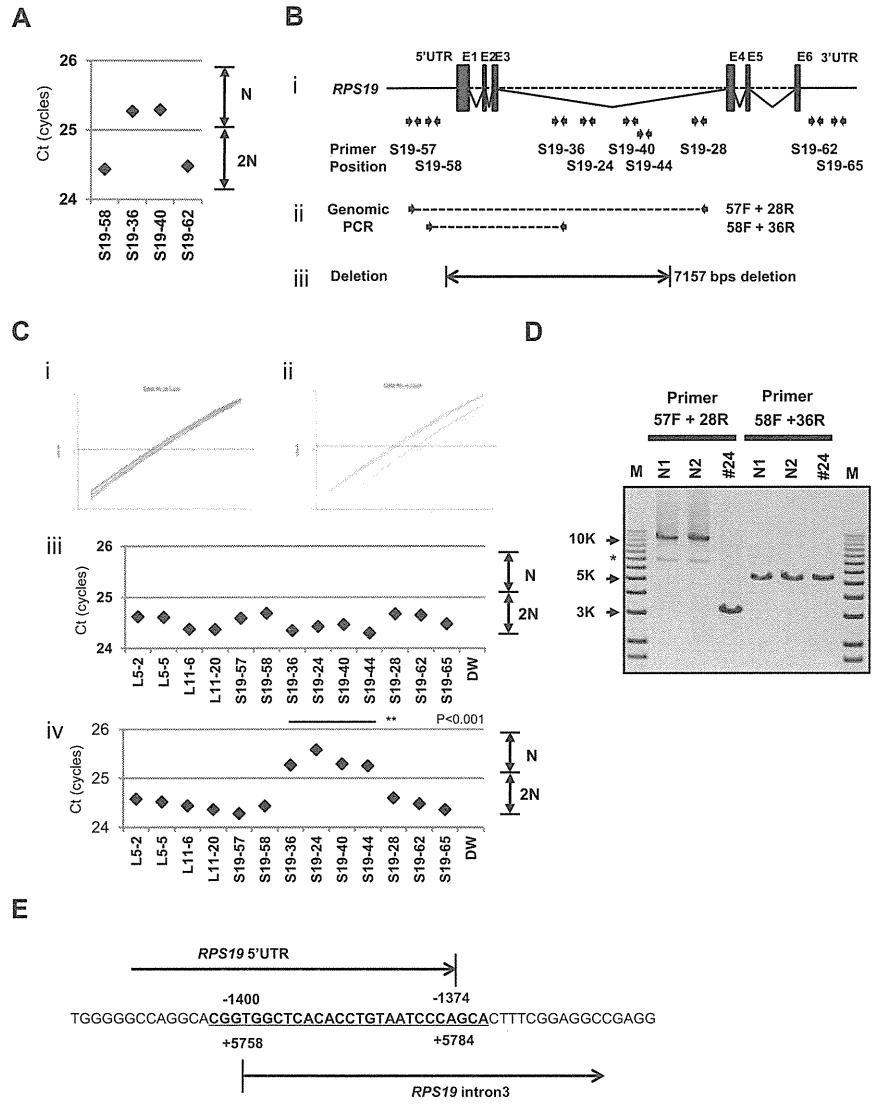


**Figure 4. Result of s-q-PCR gene copy number assay for patient 24.** (A) Results of s-q-PCR gene copy number assay for *RPS19* with 4 primer sets. (Bi) The *RPS19* gene copy number was analyzed with 9 specific primer sets for *RPS19* that span from the 5′UTR to the 3′UTR. (ii) Primer positions of genomic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

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

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

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

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

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

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

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## Authorship

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

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

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# Mutations Profile of Polycythemia Vera and Essential Thrombocythemia Among Japanese Children

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***Background.*** Acquired somatic mutations of *JAK2* have been reported to play a pivotal role in the pathogenesis of *BCR-ABL1*-negative myeloproliferative neoplasm (MPN). However, the molecular characteristics of childhood MPN remain to be elucidated. ***Patient and Methods.*** We investigated a group of pediatric patients diagnosed either with essential thrombocythemia (ET; N = 9) or polycythemia vera (PV; N = 4) according to WHO criteria (median age = 10 years; range 1.5–15 years) in whom direct sequencing was performed for the existence of genetic alterations in *JAK2, MPL, TET2, ASXL1, CBL, IDH1,* and *IDH2*. More sensitive allele specific polymerase chain reaction was used for $JAK2^{V617F}$ genotyping. ***Results.*** We found three patients harbor $JAK2^{V617F}$ mutation (2/9 ET and 1/4 PV). Bone marrow examination showed small and large megakaryocytes with dysplastic features in $JAK2^{V617F}$-positive ET patients compared to those without $JAK2^{V617F}$. We identified a previously unrecognized missense mutation at codon 1230 in exon 12 of *ASXL1* gene in ET and PV patients (1/9 ET and 1/4 PV). Otherwise, no genetic alterations could be detected in *JAK2* exon 12, *MPL, TET2, CBL, IDH1,* and *IDH2* in all ET and PV patients. ***Conclusion.*** Although *JAK2* mutations in childhood ET and PV are not as frequent as reported in adult patients, *JAK2* is the most frequently mutated gene in childhood MPN known so far. Owing to the presence of childhood MPN without any genetic alterations in *JAK2, MPL, TET2, ASXL1, CBL, IDH1,* and *IDH2*, new biological markers have to be found. Pediatr Blood Cancer 2012;59:530–535. © 2011 Wiley Periodicals, Inc.

**Key words:** *ASXL1*; essential thrombocythemia; *JAK2*; mutation; polycythemia vera

## INTRODUCTION

Polycythemia vera (PV), essential thrombocythemia (ET), and myelofibrosis (MF) are stem cell derived clonal myeloproliferative neoplasms (MPN) that result in overproduction of mature myeloid cells. Although they are recognized as a distinct clinicopathological entity, they share cardinal biological features that differentiate them from other myeloid malignancies [1]. In patients with PV there is a prevalent increase of the red cell line often associated with high granulocyte and platelet numbers, while ET is associated with an isolated elevated platelet count [2].

Genetic studies have identified that $JAK2^{V617F}$ is the most frequent mutation in BCR-ABL-negative MPN [3–8]. Analysis of *JAK2* alterations is generally accepted to be an essential component in the diagnostic criteria for PV and ET [9]. Furthermore, gain-of-function mutations in *JAK2* exon 12 and in thrombopoietin receptor (*MPL*) are observed in some patients with $JAK2^{V617F}$-negative MPN, suggesting constitutive activation of *JAK2* signaling is important in the pathogenesis of adult PV, ET, and MF [10].

Ten-eleven-translocation 2 (*TET2*) mutations were reported in various adult hematopoietic disorders including MPN [11]. It was mentioned that loss-of-function *TET2* mutations may precede or follow the acquisition of $JAK2^{V617F}$ mutation which result in accelerated cellular proliferation and contribute to the aggressive behavior of MPN [12]. Recently, mutations in Additional sex comb-like 1 (*ASXL1*), casitas B-Lineage lymphoma (*CBL*), isocitrate dehydrogenase 1 (*IDH1*) and the homologous gene *IDH2* have been described in adult MPN [13–15]. So far, the occurrence of these mutations has not been reported in childhood MPN.

Although pediatric and adult MPN exhibit similar hematologic findings, MPN in childhood is quite rare disease [3,16]. Thus, application of any diagnostic criteria developed for adult MPN would be helpful to know the biological difference between childhood and adulthood MPN [17]. In the light of this background and to better understand the molecular pathogenesis of MPN in childhood, we investigated the genetic alterations of *JAK2* ($^{V617F}$ and exon 12), *TET2* (all exons), *ASXL1* (exon 12), *CBL* (exons 7–9), *IDH1* and *IDH2* (exon 4) among PV and ET pediatric patients. Also we evaluated the occurrence of *MPL* (exon 10) mutations in *V617F*-negative ET patients.

## PATIENTS AND METHODS

### Recruitment of PV and ET Patients and Healthy Candidates

We evaluated 13 Japanese children with a diagnosis of ET (n = 9) and PV (n = 4) consecutively observed between 2005 and 2010. All patients were diagnosed in accordance to WHO criteria [18]. None of the patients had a positive family history for ET or PV. Peripheral blood and/or bone marrow samples were collected and mononuclear cell fraction was isolated and subjected to molecular analysis. Thirty healthy volunteers were

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Conflict of interest: Nothing to declare.

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recruited as controls in this study. Throughout the research, we adhered to the Japanese ethical guidelines for epidemiologic studies; and the study protocol was approved by the Institutional Review Boards of Nagoya University Graduate School of Medicine. Informed consent was obtained from the guardians of the patients following institutional guidelines.

## DNA Isolation

Mononuclear cells were separated from aspirated bone marrow and/or peripheral blood samples using a Ficoll gradient. Genomic DNA was isolated using the QIAmp DNA blood mini kit (Qiagen, Hilden, Germany) according to the manufacturer's protocol.

## Sequence Analysis

Somatic mutations in exons 12, 14 of *JAK2* [3], exon 10 of *MPL* [19], all exons of *TET2* [11], exon 12 of *ASXL1* [20], exons 7–9 of *CBL* [14], exon 4 of *IDH1* and *IDH2* [15], were searched by sequencing analysis after polymerase chain reaction (PCR) amplification of genomic DNA. PCR amplification were done in a total volume of 25 μl PCR mix containing at least 50 ng template DNA, using quick Taq PCR™ HS Dye mix (Qiagen) under the following conditions: 94°C, 2 minutes (first denaturing step); 94°C, 30 seconds; 65°C, 30 seconds; 68°C, 30 seconds to 1 minute depending on PCR product length for 35 cycles; 68°C, 7 minutes (last extension step). PCR products were purified from the reaction mixture using the QIA quick PCR purification kit (Qiagen) and directly sequenced on a DNA sequencer (ABI PRISM 3100 Genetic Analyzer Applied Biosystems, Life Technologies Japan, Tokyo) using a Big Dye terminator cycle sequencing kit (Applied Biosystems).

## Allele-Specific PCR

In combination with sequence analysis to screen $JAK2^{V617F}$ mutation efficiently, we carried out allele-specific PCR analysis according to the procedures described elsewhere [3].

# RESULTS

## Clinical and Hematological Characteristics of ET and PV Patients

Nine ET patients (five females, four males) with a median age at onset of 11.5 years (range 1.5–15 years) were included. The data of these patients are summarized in Table I. The median white blood cell counts, hemoglobin levels, and platelet counts were $10.4 \times 10^9$/L (range 5.8–19.4 $\times$ $10^9$/L), 13.3 g/dl (range 11.7–15.2 g/dl), and $1.827 \times 10^9$/L (range 923–2.900 $\times$ $10^9$/L), respectively. No clinical or laboratory evidence of chronic infections could be detected in this group of patients. Histopathological examination of bone marrow showed an elevated number of megakaryocytes in all ET patients. Interestingly, in two ET patients with $JAK2^{V617F}$ mutation (case no. 7 and case no. 11) small size and dysplastic changes of megakaryocytes (multinucleated and binucleated cells) were found, these findings raise the possibilities that $JAK2^{V617F}$ may be responsible for the distinct pathological features of bone marrow seen in those ET patients (Fig. 1). Four PV patients (two females and two males) were also evaluated in this cohort. The age at diagnosis for PV patients ranged from

**TABLE I. Clinical Characteristics of 13 Pediatric Cases With Essential Thrombocythemia and Polycythemia Vera**

| Id | S/A (Y) | D | Sp | WBC ($\times 10^9$/L) | RBCs ($\times 10^{12}$/L) | Hb (g/dl) | Ht (%) | Plt ($\times 10^9$/L) | Ph ch | *JAK2 V617F* | *JAK2* exon 12 | *MPL* exon 10 | *TET2* | *ASXL1* exon 12[a] | *CBL* | *IDH1/ IDH2* | Therapy | FUP (Y) |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| 1 | F/12 | ET | − | 10.3 | 4.2 | 11.7 | 35.3 | 238.4 | − | − | − | − | − | − | − | −/− | Aspirin | 20 |
| 2 | M/12 | ET | − | 10.4 | 4.2 | 11.8 | 35.2 | 290.0 | − | − | − | − | − | − | − | −/− | Aspirin | 15 |
| 3 | M/12 | ET | − | 9.4 | 5 | 13.3 | 41.4 | 230.6 | − | − | − | − | − | − | − | −/− | Aspirin | 13 |
| 4 | M/5 | ET | − | 19.4 | 4.9 | 12.5 | 38.2 | 182.7 | − | − | − | − | − | + | − | −/− | Aspirin | 3 |
| 5 | F/7 | PV | − | 14.6 | 5.7 | 15.8 | 46.2 | 529 | − | − | − | nt | − | + | − | −/− | — | 5 |
| 6 | M/15 | ET | + | 5.8 | 5.1 | 14 | 42.5 | 923 | − | − | − | − | − | − | − | −/− | Aspirin | 5 |
| 7 | F/8 | ET | + | 18.9 | 5.2 | 15.2 | 43.6 | 956 | − | + | − | − | − | − | − | −/− | — | 10 |
| 8 | M/15 | PV | − | 6.6 | 5.9 | 19 | 54.6 | 248 | − | − | − | nt | − | − | − | −/− | — | 4 |
| 9 | F/14 | PV | + | 16.5 | 8.5 | 16.9 | 57.8 | 114 | − | − | − | nt | − | − | − | −/− | Phlebotomy | 5 |
| 10 | M/14 | PV | − | 19.2 | 9.8 | 21.8 | 71.5 | 210 | − | + | − | nt | − | − | − | −/− | — | 7 |
| 11 | F/1.5 | ET | − | 15.2 | 5.8 | 13.5 | 40.7 | 106.4 | − | + | − | − | − | − | − | −/− | — | 1 |
| 12 | M/11 | ET | − | 10.4 | 5 | 12.9 | 39.9 | 144.8 | − | − | − | − | − | − | − | −/− | — | 1 |
| 13 | F/9 | ET | − | 14.1 | 5.1 | 13.8 | 41.9 | 245.5 | − | − | − | − | − | − | − | −/− | Aspirin | 1 |

Id, patient's number; S/A, sex/age, Y, year; D, disease; Sp, splenomegaly; WBC, white blood cell count; RBC, red blood cell count; Hb, hemoglobin; Ht, hematocrit; Plt, platelet count; Ph ch, Philadelphia chromosome; FUP, follow-up period. [a]pS1230F: possible polymorphism.

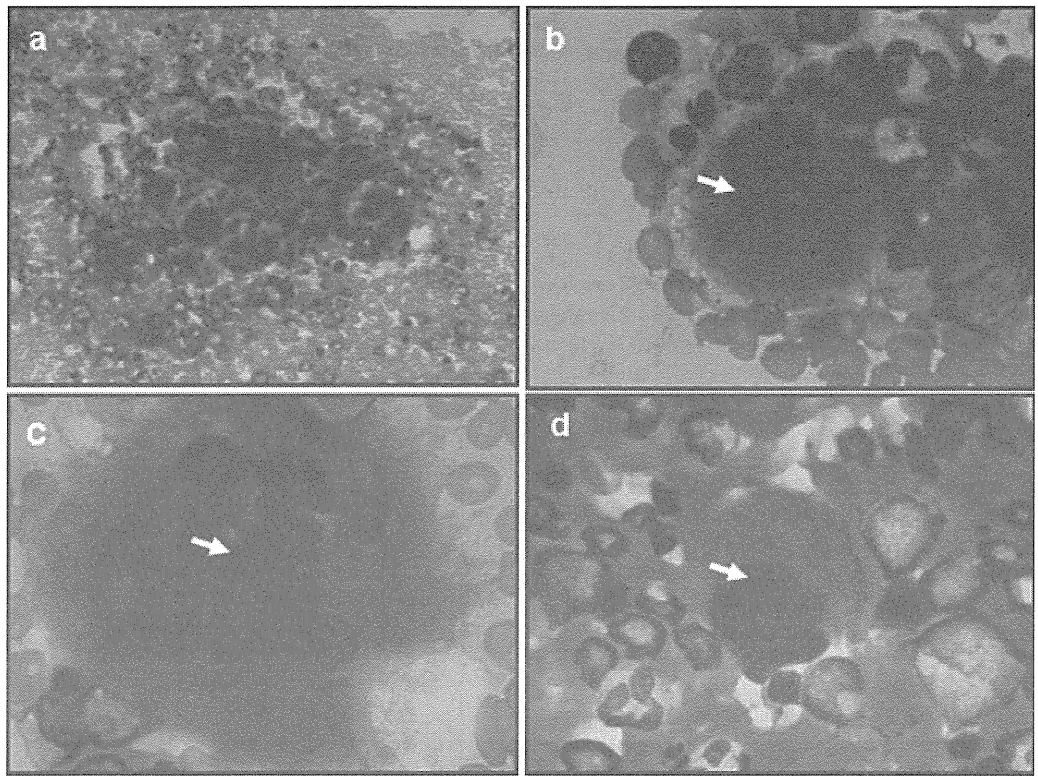


**Fig. 1.** *JAK2*$^{V617F}$-positive ET patients exhibit distinct bone marrow morphological changes. **A**: A representative of ET *JAK2*$^{V617F}$-positive cases showing hyperplasia of megakaryocytes (H&E stain ×200). **B**,**C**: Dysplastic changes of megakaryocytes (multinucleated and binucleated cells). **D**: Small sized megakaryocytes (H&E stain ×1,000).

7 to 15 years (median 14 Years). The median hemoglobin levels, white blood cell counts, and platelet counts were 19 g/dl (range 15.8–21.8 g/dl), 15.6 × $10^9$/L (range 6.7–19.2 × $10^9$/L), and 229 × $10^9$/L (range 114–529 × $10^9$/L), respectively. Cytogenetic analysis of bone marrow revealed normal karyotype in all ET and PV patients.

### Mutations of Candidate Genes in Pediatric ET and PV Patients

We analyzed the sequences of several candidate genes in ET and PV patients. Through *allele specific* PCR combined with sequence analysis, a G → T alteration was detected at codon 617 in exon 14 of *JAK2* (which results in a substitution of valine for phenylalanine) in two out of nine (22%) ET patients and one out of four (25%) PV patients (Fig. 2; Table I). We also found a point mutation (C to T) at codon 1230 in exon 12 of *ASXL1* in one (11%) out of nine ET patients and in one (25%) out of four PV patients, which results in amino acid conversion from serine to phenylalanine. This mutation was not included in the public database (Fig. 3) (Table I), but 3 out of 30 healthy Japanese control were found to harbor this mutation, indicating that *ASXL1* (p.S1230F) mutation could be encountered as SNP. The occurrence of *TET2* alterations was also investigated, synonymous point mutations c.3117G>A (S1039S) and c.4140T>C (H1380H) were detected in ET patients and confirmed in healthy controls. After exclusion of common SNPs present in public databases and/or detected in 30 healthy controls survey, no other *TET2* mutations could be found in ET and PV patients. None of the ET and PV patients showed genetic alterations in *JAK2* exon 12, *CBL*, *IDH1*, and *IDH2* genes. No *MPL* mutations were detected among ET pediatric patients included in this cohort (Table I).

## DISCUSSION

An increasing interest in *BCR-ABL1*-negative MPN in childhood has emerged recently, with a particular emphasis on ET and PV. Considering the low incidence of ET and PV among children compared to the adult patients, we believe that the new biological insights will help us to validate new diagnostic and therapeutic guidelines for childhood MPN. We reported nine cases of ET with median age 11.5 years (range 1.5–15 years) and high platelet counts (median: 1.827 × $10^9$/L; range: 923–2.900 × $10^9$/L). *JAK2*$^{V617F}$-positive ET patients had a predominant increase in the leucocytic counts. Six out of nine ET patients received antiplatelet drug (Aspirin) (Table I). Four patients with PV were also included in this study; the age at diagnosis for those patients ranged from 7 to 15 years (median 14 years) presented with high hematocrit values (range 46.2–71.5%). The PV patient who had a *JAK2*$^{V617F}$ mutation, showed a significant increase in hemoglobin, hematocrit level, and WBCs counts compared to *JAK2*$^{V617F}$-negative PV patients. Phlebotomy was performed for this patient in order to remove the excess cellular elements and to decrease the hematocrit value, while the other three patients with PV were not given any therapy (Table I). These data are consistent with previous reports mentioned that most children with ET

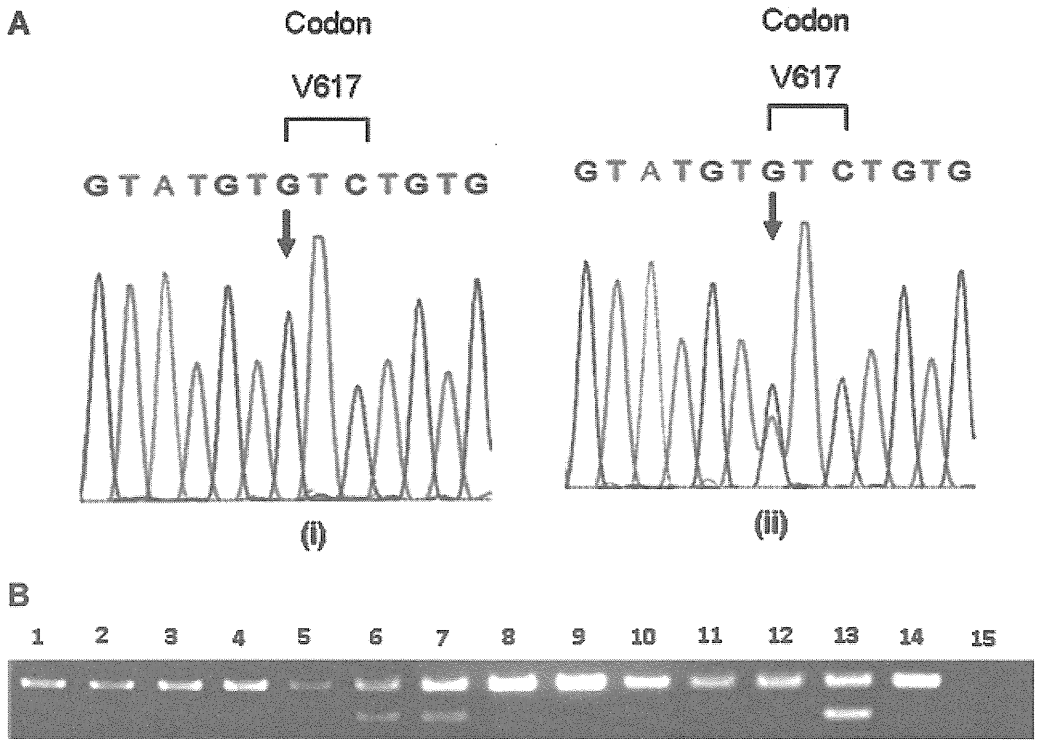


**Fig. 2.** *JAK2*$^{V617F}$ mutation among PV and ET pediatric patients. **A**: Sequence traces. Showing mutation analysis of JAK2 gene (i) Nucleotide sequences and corresponding amino acid sequences of *JAK2* wild-type (ii) G to T JAK2 mutation in two ET patients and one PV patient. Arrows directed to the relevant base. **B**: Allele-specific PCR displayed results of ET and PV cases. A 203-bp (lower band) indicates mutant DNA with *JAK2*$^{V617F}$ mutation while a 364-bp (upper band) indicates wild-type and used as internal control. Lanes 1–9: ET cases; lanes 10–13: PV cases; lane 14: healthy volunteer (negative control); lane 15: no template; lanes 6, 7, and 13: V617F-positive patients.

are characterized by young age and high platelet counts, nevertheless, almost all PV pediatrics patients presenting with high hematocrit levels [21,22].

Identification of *JAK2*$^{V617F}$ as a biological marker for PV and ET has improved our understanding of the molecular pathogenesis in these disorders. Previous studies of *JAK2*$^{V617F}$ have indicated that its prevalence within childhood ET is variable [16,21,23]. In agreement with Randi's report [23] we showed that 22% of pediatric patients with ET carried *JAK2*$^{V617F}$ mutation. On the other hand, we found that 25% of PV patients had *JAK2*$^{V617F}$, consistently with that reported by Teofili et al. [16]. In addition to the sequencing method used in this study, we confirmed *JAK2*$^{V617F}$ mutation by allele-specific PCR, which is considered a highly sensitive technique to detect this mutational type [24]. In a previous study employing the same technique [16], a higher frequency of *JAK2*$^{V617F}$ mutation was detected in 7 cases (39%) of 18 sporadic pediatric ET patients. In line with other observations [21,23], our results showed that childhood incidence of *JAK2*$^{V617F}$ mutation in ET and PV is lower than adulthood forms. Interestingly, *JAK2*$^{V617F}$-positive ET patients exhibit distinct histopathological changes of their bone marrow compared to *JAK2*$^{V617F}$-negative patients (who showed only an elevated number of megakaryocytes) including the presence of megakaryocytes with small size and dysplastic features (multinucleated and binucleated form), implying that it might possible to improve the diagnostic ability by integrated classification scheme including the molecular and bone marrow histopathologic findings. However, these provisional speculations should be confirmed in the subsequent cohorts.

Differently from adult patients, we could not find any alteration in exon 12 of *JAK2* among V617F-negative MPN pediatrics population, while another report showed that two patients had *JAK2* exon 12 out of eight pediatric PV cases [25].

Previous data suggested that *TET2* mutation could be acquired before *JAK2*$^{V617F}$ in adult MPN [11]. Tefferi [26] reported that *CBL* mutation might occur in post-ET/PV fibrotic phase, while *IDH* mutations are relatively frequent in blast- but not in chronic-phase of MPN. Our cohort shows that no patient (ET or PV) harbors, *CBL*, *IDH1*, *IDH2*, and *MPL* mutations, suggesting these genetic alterations are relatively rare to occur in childhood MPN. In our study, none of the patients had a positive family history for ET or PV. However, it has been recently demonstrated that patients diagnosed as familial MPN with thrombocytosis might harbor *JAK2* or *MPL* mutations which are not considered as the primary pathogenetic lesions. By contrast, alterations of *MPL* or thrombopoitein (*THPO*) genes are diseases causing defects in hereditary thrombocytosis [27]. The molecular delineation of PV revealed that *JAK2*$^{V617F}$ mutation has a pathohgenetic role in this disorder. Nevertheless, primary familial congenital polycythemia is caused by mutations in the Epo receptor (EPOR) gene [28]. Moreover, alteration of oxygen-sensing pathway has been shown to be involved in the pathogenesis of the congenital forms of erythrocytosis [29]. Specifically, mutations in the genes encoding VHL, PHD2, and

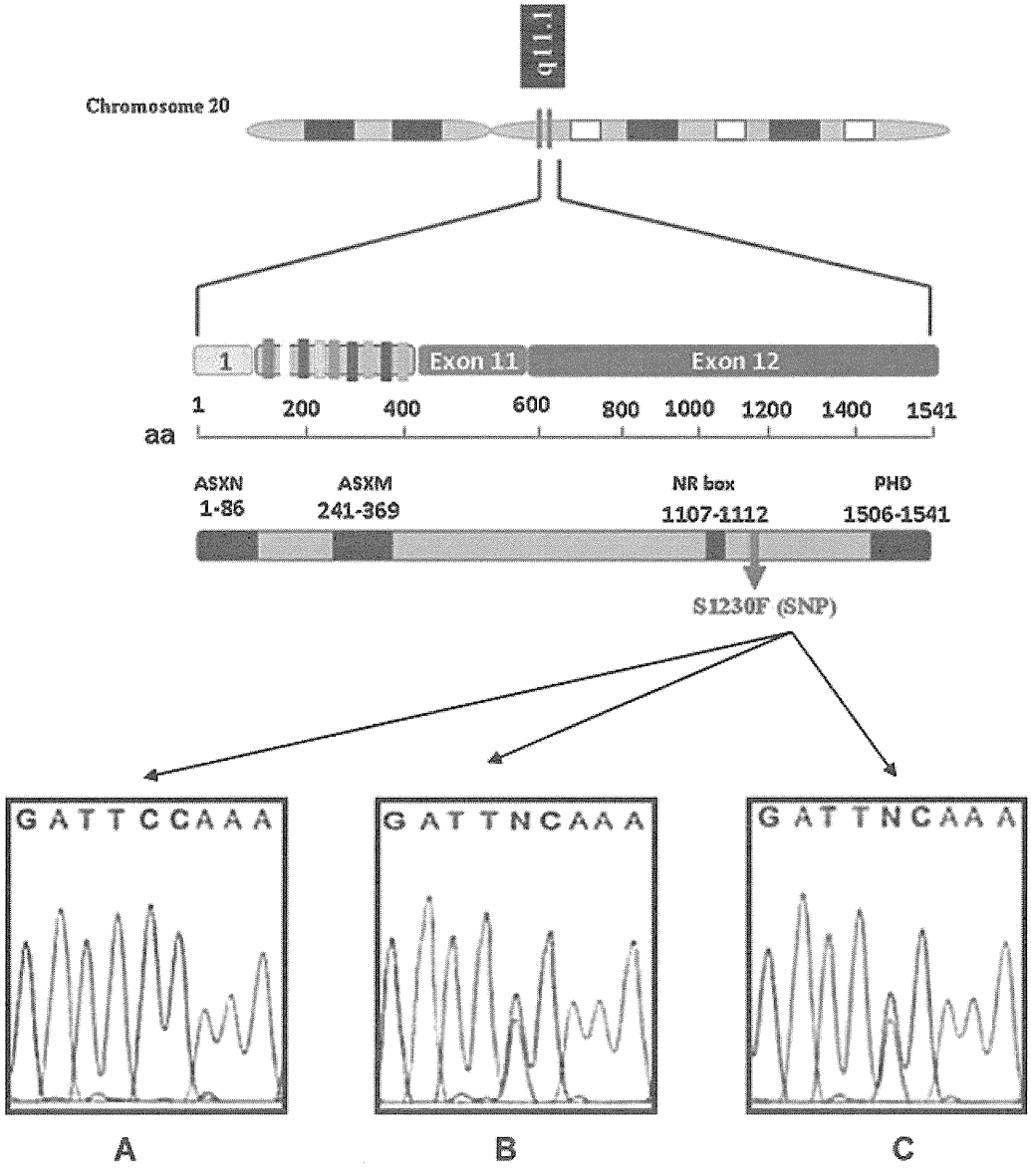


**Fig. 3.** Schematic representation of *ASXL1* showing its chromosomal location, principle functional, and conserved domains. A missense p.S1230F mutation is indicated by red vertical arrows. Nucleotide sequence showing wild-type (**A**) and mutant *ASXL1* in ET (**B**) and PV (**C**) patients.

HIF-2α have been detected in patients with familial erthrocytosis [30–32].

Here, we have described a missense mutation at codon 1230 in exon 12 of *ASXL1* gene in ET and PV patients that has not been reported before (Fig. 3). We have identified this mutation in healthy Japanese volunteers. Therefore, we conclude that this variation represents a SNP. Previous studies on adulthood MPN showed that *ASXL1* mutation was found in association with $JAK2^{V617F}$ mutation in PV [33] and was identified as a sole lesion in ET [13]. Large cohort is highly required to better define the occurrence of other genetic alterations that might contribute to the pathophysiology of childhood MPN. In conclusion, our findings provide further evidence that *JAK2* mutations in childhood ET and PV are not as frequent as reported in adulthood ET and PV. Given the presence of MPN without *JAK2/MPL/ASXL1/TET2/IDH1/IDH2* mutations, it indicates the existence of other alternative unidentified mutations which may contribute to the pathogenesis of childhood MPN.

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# Acceptable HLA-mismatching in unrelated donor bone marrow transplantation for patients with acquired severe aplastic anemia

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**We retrospectively analyzed the effect of HLA mismatching (HLA-A, -B, -C, -DRB1, -DQB1) with molecular typing on transplantation outcome for 301 patients with acquired severe aplastic anemia (SAA) who received an unrelated BM transplant through the Japan Marrow Donor Program. Additional effect of HLA-DPB1 mismatching was analyzed for 10 of 10 or 9 of 10 HLA allele-matched pairs (n = 169). Of the 301 recipient/donor pairs, 101 (33.6%) were completely matched at 10 of 10 alleles, 69 (23%) were mismatched at 1 allele, and 131 (43.5%) were mismatched at ≥ 2 alleles. Subjects were classified into 5 subgroups: complete match group (group I); single-allele mismatch group (groups II and III); multiple alleles restricted to HLA-C, -DRB1, and -DQB1 mismatch group (group IV); and others (group V). Multivariate analysis indicated that only HLA disparity of group V was a significant risk factor for poor survival and grade II-IV acute GVHD. HLA-DPB1 mismatching was not associated with any clinical outcome. We recommend the use of an HLA 10 of 10 allele-matched unrelated donor. However, if such a donor is not available, any single-allele or multiple-allele (HLA-C, -DRB1, -DQB1) mismatched donor is acceptable as an unrelated donor for patients with severe aplastic anemia. (*Blood*. 2011;118(11):3186-3190)**

## Introduction

BM transplantation from an unrelated donor (UBMT) is indicated as salvage therapy for patients with severe aplastic anemia (SAA) who fail to respond to immunosuppressive therapy. Early results of UBMT have not been encouraging because of a high incidence of graft failure and GVHD.[1-3] The Center for International Blood and Marrow Transplant Research (CIBMTR) reported the outcome of 232 patients with SAA who received an UBM transplant between 1988 and 1998.[3] The 5-year probabilities of overall survival (OS) were 39% and 36% after matched unrelated and mismatched unrelated donor transplantations, respectively. We previously reported the outcome of 154 patients with SAA who received an UBM transplant between 1993 and 2000 through the Japan Marrow Donor Program (JMDP).[4] The 5-year OS rate was 56% in that study.

In several recent studies, the effect of HLA high-resolution matching on outcome of patients who received an UBM transplant has been elucidated.[5-8] However, results have been derived primarily from an analysis of patients with hematologic malignancies. Major obstacles for UBMT are different between patients with hematologic malignancies and patients with SAA. Relapse is a main cause of death for patients with hematologic malignancies, and GVL effect may result in decrease of relapse rate. In contrast, graft failure is the main problem, and GVHD is the only negative effect for patients with SAA. Therefore, optimal HLA matching may be different between these 2 populations. Algorithms for donor selection derived from an analysis of patients with hematologic malignancies might not be useful for patients with SAA. However, a few studies have focused on the clinical significance of HLA-allele compatibility in patients with SAA.[2,4,9,10]

In a previous study, we analyzed the clinical significance of HLA allele mismatching in 142 patients with SAA, in whom data of high-resolution typing of HLA-A, -B, and -DRB1 were available.[4] Mismatching of HLA-A or -B alleles between donor and recipient was a strong risk factor for acute and chronic GVHD and OS, whereas mismatching of the HLA-DRB1 allele did not have a significant effect on patient outcomes. In the study from the National Marrow Donor Program, mismatching of HLA-DRB1 was the most crucial risk factor for OS.[2] These results indicate that better donor selection through high-resolution typing might result in improved outcome in patients with SAA who receive an UBM transplant. In fact, several recent studies showed a significantly improved outcome in patients with SAA who received and UBM transplant over time.[11,12] In particular, better HLA matching by high-resolution typing has been thought to contribute to these improvements.[4,9-11]

On the contrary, restricting BMT to donor-recipient pairs perfectly matched at high-resolution typing reduces the chance of undergoing UBMT for many patients. Therefore, strategies for selecting a partially HLA allele mismatched donor are required when a full matched donor cannot be identified. Here, we report a detailed analysis of outcome in 301 patients with SAA who were

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typed for HLA-A, -B, -C, -DRB1, -DQB1, and -DPB1 by a molecular technique and underwent UBMT through the JMDP.

## Methods

### Patients

From February 1993 to April 2005, 380 consecutive patients with acquired SAA received an UBM transplant through the JMDP. Patients with inherited AA, such as Fanconi anemia, and patients who received a BM transplant > 2 times were excluded. This study includes 301 patients in whom molecular analysis of HLA-A, -B, -C, DRB1, and -DQB1 were performed by DNA-based methods. HLA-DPB1 was analyzed in 299 of these patients. The previous study included 142 patients in whom molecular typing was performed only for HLA-A, -B, and -DRB1.

Characteristics of the 301 patients and donors are shown in Table 1. Briefly, patients (173 males and 128 females) were between birth and 64 years of age (median, 17 years of age). The median disease duration before BMT was 43 months (range, 4-436 months). All patients failed conventional immunosuppressive therapies and were considered candidates for UBMT. All patients or their guardians gave informed consent for transplantation and submission of the data to the JMDP.

### Transplantation procedure

Characteristics of the transplantation procedures are also shown in Table 1. Patients underwent transplantations at individual centers following the local protocols for preconditioning regimens and GVHD prophylaxis. The various preconditioning regimens used by individual centers were classified into 5 categories: TBI or LFI + CY + ATG (n = 128), TBI or LFI + CY (n = 103), TBI or LFI + CY + Flu with or without ATG (n = 39), CY + Flu + ATG (n = 8), and others (n = 23). In 130 patients, CsA and MTX were used for prophylaxis against GVHD; 134 patients received FK instead of CsA. The remaining 35 patients received other GVHD prophylaxis. Ex vivo T-cell depletion was not used for any patient. The median number of infused nucleated marrow cells was $3.1 \times 10^8$/kg. One-half (n = 150) of the transplantations were performed before 2000, and 151 were done after 2001.

### HLA typing and definition of mismatching

HLA matching between patients and donors was based on HLA serotyping according to the standard technique. Partial HLA-A and -B alleles and complete HLA-DRB1 alleles were identified as confirmatory HLA typing during the coordination process, and HLA-A, -B, -C, -DQB1, and -DPB1 alleles were retrospectively reconfirmed or identified after transplantation. Molecular typing of HLA-A, -B, -C, -DQB1, -DRB1, and -DPB1 alleles was performed by the Luminex microbead method (Luminex 100 system) adjusted for the JMDP and in part by the sequencing-based typing method. Mismatching was defined as the presence of donor antigens or alleles not shared by the recipient (rejection vector) or the presence of recipient antigens or alleles not shared by the donor (GVHD vector).

### Definition of transplantation-related events

The day of engraftment was defined as the first day of 3 consecutive days on which neutrophil count exceeded $0.5 \times 10^9$/L. Patients who did not reach neutrophil counts > $0.5 \times 10^9$/L for 3 consecutive days after transplantation were considered to have primary graft failure. Patients with initial engraftment in whom absolute neutrophil counts declined to < $0.5 \times 10^9$/L subsequently were considered to have secondary graft failure. Acute GVHD was evaluated according to standard criteria in patients who achieved engraftment, and chronic GVHD was evaluated according to standard criteria in patients who achieved engraftment and survived > 100 days after transplantation.

### Data collection and statistical analysis

Transplantation data were collected with the use of standardized forms provided by the JMDP. Patient baseline information and follow-up reports were submitted at 100 days and annually after transplantation. Analysis of patient outcome was performed with the date of last reported follow-up or date of death. Data were analyzed as of July 1, 2007.

Probability of OS and 95% confidence interval (95% CI) were estimated from the time of transplantation according to the Kaplan-Meier method. Cumulative incidence of neutrophil engraftment at day 42 was analyzed in the whole of patients by treating deaths until day 42 as a competing risk. Cumulative incidence of acute GVHD at day 100 was analyzed in patients who sustained engraftment by treating deaths until day 100 as a competing risk. Cumulative incidence of chronic GVHD at day 365 was analyzed in patients who sustained engraftment and survived longer than day 100 by treating deaths until day 365 as a competing risk. In univariate analysis, the log-rank test or Gray test was used to assess the significance of HLA allele mismatching on clinical outcomes. The Mann-Whitney *U* test was used to compare the median days of neutrophil engraftment. The chi-square test or Mann-Whitney *U* test was used to compare patient characteristics and transplantation procedures between the patient groups. All *P* values < .05 were considered statistically significant, whereas *P* values between .05 and .1 were considered as marginally significant.

Multivariate analyses were performed to assess the effect of HLA allele mismatching on the clinical outcome by Cox proportional hazard model (each mismatched group vs fully matched group; hazard risk = 1.0 as a reference group). Factors other than HLA mismatching included in the models were patient age, patient sex, donor age, donor sex, disease duration before BMT, infused cell dose, matching of ABO blood type, GVHD prophylaxis, and preconditioning regimens.

## Results

### HLA matching by DNA typing

Of the 301 recipient/donor pairs, 101 pairs (33%) were completely matched at HLA-A, -B, -C, -DRB1, and -DQB1 allele; 69 pairs (23%) were mismatched at 1 HLA allele; 59 pairs (20%) were mismatched at 2 HLA alleles; and 72 pairs (24%) were mismatched at ≥ 3 alleles (Table 2). The number and frequency of 1-allele and 2-allele mismatches in either GVHD or rejection vector or both vectors in each HLA allele were 55 (18.3%) and 7 (2.3%) in HLA-A allele, 32 (10.6%) and 2 (0.7%) in HLA-B allele, 130 (43.2%) and 10 (3.3%) in HLA-C allele, 68 (22.6%) and 5 (1.7%) in HLA-DRB1 allele, 80 (26.6%) and 13 (4.3%) in HLA-DQB1 allele, and 179 (59.5%) and 44 (14.6%) in HLA-DPB1 allele, respectively. Because the frequency of mismatching was too high at the DPB1 allele, analysis of DPB1 mismatching was separated from that of other alleles. In addition, because the number of single-allele mismatched pairs of HLA-A, -B, -C, -DRB1, and -DQB1 were too small for separate analyses, HLA-A and -B were grouped into the mismatch of the HLA-A or HLA-B allele (A/B) and HLA-DRB1 and -DQB1 into the mismatch of the HLA-DRB1 or HLA-DQB1 allele (DRB1/DQB1), respectively.

### Survival

Of the 301 patients, 202 are alive at the time of analysis with an observation time from 3 to 128 months (median, 44 months) after transplantation. Five-year OS was 66.3% (95% CI, 60.7%-72.5%) in the whole population (supplemental Figure 1, available on the *Blood* Web site; see the Supplemental Materials link at the top of the online article). Subgroup analyses were performed in 8 main subgroups (> 15 recipients) as follows: (1) complete match group (n = 101), (2) single locus (A/B) mismatch group (n = 20), (3) single (C) mismatch group (n = 42), (4) 2 loci (A/B + C) mismatch group (n = 20), (5) 2 loci (DRB1/DQB1) mismatch group (n = 19), (6) 3 loci (A/B + C) mismatch group (n = 15), (7) 3 loci (C + DRB1/DQB1) mismatch group (n = 29), and

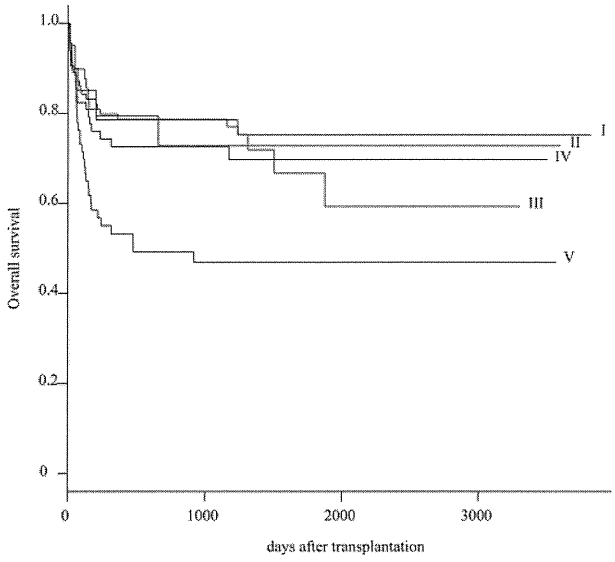


**Figure 1. Kaplan-Meier estimates of OS in 5 HLA groups.**

(8) 3 loci (A/B + C + DRB1/DQB1) mismatch group (n = 21). OS was significantly worse in the following groups than in the complete match group (75.2%): 2 loci (A/B + C) mismatch group (49.0%; $P = .022$), ≥ 3 loci (A/B + C) mismatch group (40.0%; $P = .002$), and A/B + C + DRB1/DQB1 mismatch group (56.1%; $P = .031$; supplemental Table 1).

On the basis of these primary results, 301 patients were reclassified into 5 subgroups: HLA complete match group (group I; n = 101), single-allele (A/B) mismatch group (group II; n = 20), single-allele (C or DRB1/DQB1) mismatch group (group III; n = 49), multiple-allele (restricted to C or DRB1/DQB1) mismatch group (group IV; n = 68), and others (group V; n = 63). The probability of OS at 5 years was 75.2% (95% CI, 84.8%-66.7%) in group I, 72.7% (95% CI, 96.7%-54.7%) in group II, 66.7% (95% CI, 85.1%-52.3%) in group III, 69.7% (95% CI, 82.6%-58.8%) in group IV, and 46.8% (95% CI, 61.7%-35.5%) in group V, respectively (Table 3; Figure 1). Survival rate was significantly inferior in group V than in group I ($P = .003$).

To avoid or minimize the effect of other HLA alleles mismatching, the effect of HLA-DPB1 mismatching was evaluated in group I (n = 101) and groups II + III (n = 69), independently. HLA-DPB1 was matched in 51 recipient/donor pairs (30%) and mismatched in 118 pairs (70%). Patient characteristics and transplantation procedures were not different between HLA-DPB1 matched and mismatched groups (supplemental Table 2). The probability of OS at 5 years in group I was equivalent between the HLA-DPB1 matched group (74.4%; 95% CI, 93.2%-59.4%) and the HLA-DPB1 mismatched group (75.7%; 95% CI, 87.2%-65.8%; $P = .894$; Table 4; Figure 2A). It was also equivalent in groups II + III (71.4%; 95% CI, 93.6%-54.5% in the HLA-DPB1 matched group and in the HLA-DPB1 mismatched group (67.1%; 95% CI, 85.6%-52.5%; $P = .826$; Table 4; Figure 2B). Multivariate analysis identified significant unfavorable variables as follows: recipient age (0-10 years: relative risk [RR] = 1.0; 11-20 years: RR = 4.092, $P = .002$; 21-40 years: RR = 3.970, $P = .004$; > 41 years: RR = 5.241, $P = .003$), conditioning regimen (Flu + CY + TBI/LFI ± ATG: RR = 1.0; CY + TBI/LFI: RR = 4.074, $P = .058$; others: RR = 6.895, $P = .013$), HLA mismatching (group I: RR = 1.0; group V: RR = 1.967, $P = .023$), donor sex (female: RR = 1.0; male: RR = 1.850, $P = .016$), and GVHD prophylaxis (FK + MTX: RR = 1.0; other: RR = 1.754, $P = .024$), blood type (ABO match or minor mismatch: RR = 1.0; major mismatch or bidirection: RR = 1.948, $P = .005$), and disease duration (< 7 years: RR = 1.0; > 7 years: RR = 1.540, $P = .084$; Table 5).

### Engraftment

The cumulative incidence of neutrophil engraftment at day 42 was evaluated in 300 patients. It was 90.3% (95% CI, 93.7%-86.9%) in the whole population. Subgroup analyses showed that it was 93.0% (95% CI, 98.2%-87.8%) in group I, 90.0% (95% CI, 100%-74.6%) in group II, 89.8% (95% CI, 98.9%-80.7%) in group III, 92.6% (95% CI, 99.2%-86.0%) in group IV, and 84.1% (95% CI, 93.4%-74.8%) in group V ($P = .185$; Table 3). The median time to engraftment was 17 days in group I; 18 days in groups II, III, and IV; and 19 days in group V. Engraftment was marginally delayed in group V compared with group I ($P = .053$). Additional HLA-DPB1 mismatching did not affect the cumulative incidence of engraftment in the 10 of 10 and 9 of 10 matched groups, respectively (Table 4). In multivariate analysis, blood type (ABO match or

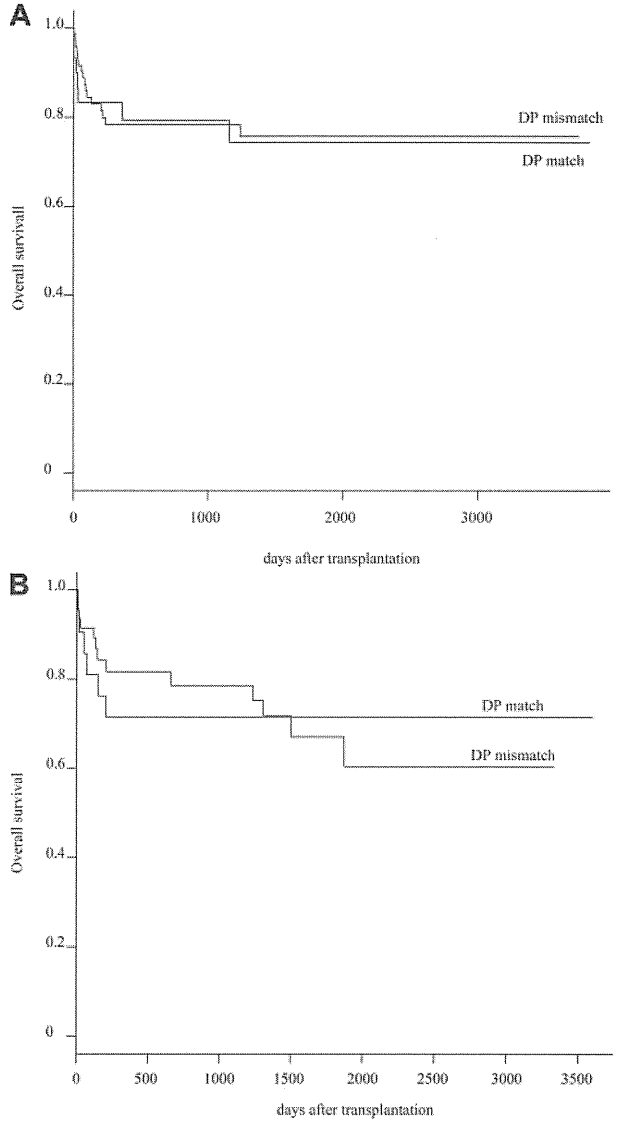


**Figure 2. OS between HLA-DPB1 matched group and HLA-DPB1 mismatched group.** (A) Difference of OS between HLA-DPB1 matched group and HLA-DPB1 mismatched group in 10 of 10 HLA allele matched pairs. (B) Difference of OS between HLA-DPB1 matched group and HLA-DPB1 mismatched group in 9 of 10 HLA allele matched pairs.

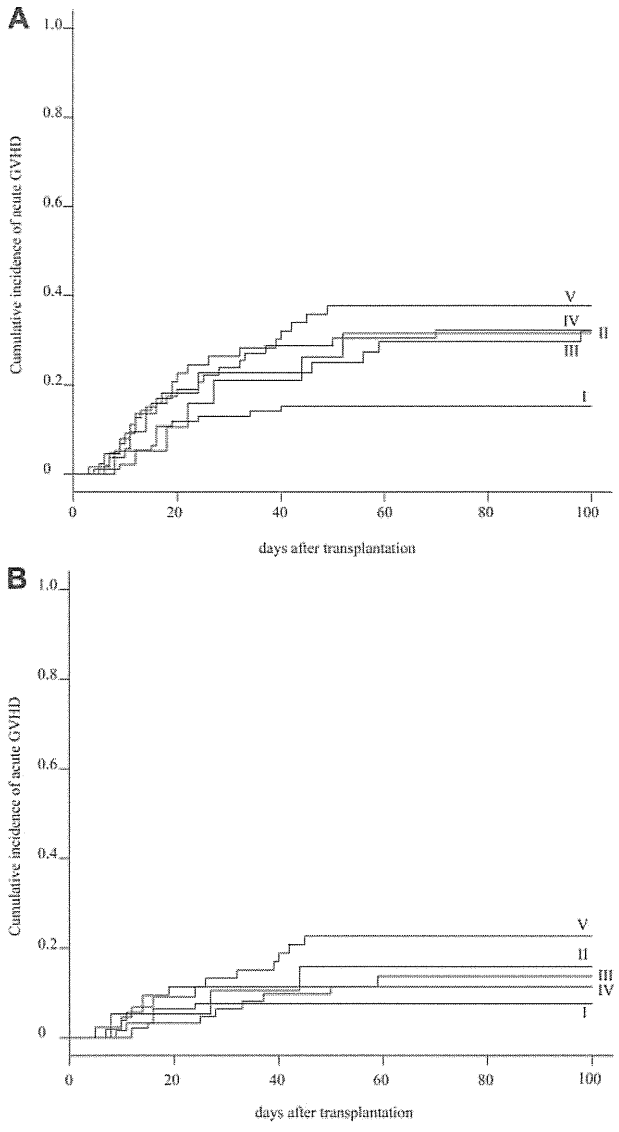


**Figure 3. Cumulative incidence of acute GVHD.** (A) Cumulative incidence of grade II-IV acute GVHD in 5 HLA groups. (B) Cumulative incidence of grade III-IV acute GVHD in 5 HLA groups.

minor mismatch: RR = 1.0; major mismatch or bidirection pair: RR = 5.102, $P$ = .039) and HLA mismatching (group I: RR = 1.0; group V: RR = 4.906, $P$ = .035) were significant risk factors for engraftment.

### Acute GVHD

The cumulative incidence of acute GVHD at day 100 was evaluated in 272 patients. The cumulative incidence of grade II-IV and grade III-IV acute GVHD was 27.2% (95% CI, 32.5%-21.9%) and 12.9% (95% CI, 16.9%-8.9%) in the whole population, respectively (supplemental Figure 2). Subgroup analyses showed that the cumulative incidence of grades II-IV acute GVHD was statistically lower in group I (15.1%; 95% CI, 22.4%-7.8%) than in group V (37.7%; 95% CI, 50.9%-24.5%; $P$ = .037), and marginally lower than in group III (31.8%; 95% CI, 45.8%-17.8%) and group IV (31.7%; 95% CI, 43.3%-20.1%; Table 3; Figure 3A). Whereas the cumulative incidence of grade III-IV acute GVHD was not significantly different among 5 groups: 7.5% (95% CI, 24.6%-0%) in group I, 15.8% (95% CI, 32.7%-0%) in group II, 13.6% (95% CI, 23.9%-3.3%) in group III, 11.1% (95% CI, 18.9%-3.3%) in group IV, and 22.6% (95% CI, 34.0%-11.2%) in group V ($P$ = .139; Table 3; Figure 3B). Additional HLA-DPB1 mismatching evaluated in 155 patients did not affect the cumulative incidence of grade II-IV acute GVHD in the 10 of 10 and 9 of 10 matched groups, respectively (Table 4). Multivariate analysis showed that a significantly higher incidence of grade II-IV acute GVHD was associated with HLA mismatching (group I: RR = 1.0; group III: RR = 3.975, $P$ = .002; group IV: RR = 3.334, $P$ = .004; group V: RR = 3.665, $P$ = .002). Other significant risk factors were the preconditioning regimen (Flu +CY + TBI/LFI ± ATG: RR = 1.0; TBI/LFI + CY: RR = 5.224, $P$ = .003), and donor sex (female: RR = 1.0; male: RR = 1.844, $P$ = .034; supplemental Table 3).

### Chronic GVHD

The cumulative incidence of chronic GVHD at day 365 was evaluated in 232 patients. It was 24.5% (95% CI, 30.3%-18.7%) in the whole population. Subgroup analyses showed that it was comparable among the 5 HLA groups: 19.8% (95% CI, 28.8%-10.8%) in group I, 26.3% (95% CI, 49.3%-3.3%) in group II, 28.2% (95% CI, 43.3%-13.1%) in group III, 26.9% (95% CI, 39.2%-14.6%) in group IV, and 27.3% (95% CI, 42.1%-12.5%) in group V ($P$ = .922; Table 3; supplemental Figure 3). HLA-DPB1 mismatching did not affect the cumulative incidence of chronic GVHD (Table 4).

## Discussion

The survival rate in UBMT has increased substantially over the past 10 years in patients with SAA.[8-15] A 5-year survival rate of 90% has been reported in a small series of children.[16,17] A recent meta-analysis showed that detailed HLA-matching facilitated by DNA-based typing has contributed to the improved survival rate in patients with SAA who received an UBM transplant.[18] However, many patients with SAA who need hematopoietic stem cell transplantation do not have an HLA-complete matched donor. Our multivariate analysis indicated that among 4 HLA-mismatched groups, only HLA disparity of group V was a statistically significant unfavorable variable. We conclude that any type of HLA single-allele mismatch or multiple-allele mismatch within HLA-C and HLA class II (DRB1 or DQB1) is acceptable as an unrelated donor when an HLA complete match donor is unavailable.

We previously reported that HLA class I allele mismatching (HLA-A or -B) but not class II allele (HLA-DRB1) mismatching was a significant risk factor for survival when 6 alleles were analyzed.[4] HLA-A or -B mismatching pairs in the previous study were separated into 2 groups in the current study in which 10 alleles were analyzed. One group was a true single-allele mismatching pair of HLA-A or -B alleles (group II), and another was a multiple-allele mismatching pair of HLA-A or -B plus HLA-C and/or class II HLA alleles (group V). Because HLA-C and -DQB1 alleles were not typed, this type of multiple-allele mismatching might be mistaken as a single-allele mismatching pair, which was the reason for the inferior outcome of HLA-class I mismatching pairs in our previous study.

As the same in our previous study, mismatching of HLA-DRB1 did not provide a significant impact on clinical outcome. An HLA-DRB1 mismatching pair was also classified into a true single-allele mismatching of HLA-DRB1 (group III) and HLA-DRB1 plus HLA-C and/or HLA-DQB1 mismatching pairs (group IV). Interestingly, multiple mismatching of group IV was not

associated with increased mortality, which may explain why mismatching of HLA-DRB1 did not have a deleterious effect in the previous study.

The effect of HLA-DPB1 mismatching was also evaluated in HLA complete matched pairs (n = 101) and single-allele mismatched pairs (n = 69). The importance of DPB1 matching in the UBMT setting has been mainly discussed in patients with hematologic malignancies. Although results were controversial in early reports, recent studies support a significant effect of DPB1 mismatching on the incidence of acute GVHD, disease relapse, and OS.[19-22] In a large dataset of the International Histocompatibility Working Group, there was a statistically significant higher risk of both grade II-IV and grade III-IV acute GVHD.[19] The increased risk of acute GVHD was accompanied by a statistically significant decrease in disease relapse, probably because of the GVL effect, which offset the deleterious effect of acute GVHD. Survival rate was significantly better in DPB1-matched transplantations in patients with standard-risk leukemia but not in advanced leukemia. Conversely, in the HLA-mismatched group, there was a significant survival advantage in DPB1 mismatched pairs.

We expected that DPB1 matching might be beneficial for patients with AA who do not need the GVL effect. However, clinical outcomes, including incidence of acute GVHD, were not affected by DPB1 mismatching. HLA-DPB1 typing may not be essential to the donor selection algorithm for patients with SAA. Indeed, HLA-DPB1 mismatching was observed in 74% of recipient/donor pairs, and it may be practically difficult to find HLA 12 of 12 matched donors.

In conclusion, this retrospective study confirms the importance of HLA matching between recipients and donors to improve the outcome of UBMT for patients with SAA patients. However, this study showed that only 33% of patients received transplants from an HLA 10 of 10 matched donor. The availability of unrelated hematopoietic stem cell transplants can be increased through the judicious selection of donors with HLA mismatches that do not substantially lower survival.

## Authorship

Contribution: H. Yagasaki analyzed the data and wrote the paper; S. Kojima designed the research and analyzed the data; and H. Yabe, K.K., H.K., H.S., M.T., S. Kato, T.K., Y.M., and Y.K performed and supervised the research.

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# Copy Number Variations Due to Large Genomic Deletion in X-Linked Chronic Granulomatous Disease

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## Abstract

Mutations in genes for any of the six subunits of NADPH oxidase cause chronic granulomatous disease (CGD), but almost 2/3 of CGD cases are caused by mutations in the X-linked *CYBB* gene, also known as NAD (P) H oxidase 2. Approximately 260 patients with CGD have been reported in Japan, of whom 92 were shown to have mutations of the *CYBB* gene and 16 to have chromosomal deletions. However, there has been very little detailed analysis of the range of the deletion or close understanding of the disease based on this. We therefore analyzed genomic rearrangements in X-linked CGD using array comparative genomic hybridization analysis, revealing the extent and the types of the deletion genes. The subjects were five Japanese X-linked CGD patients estimated to have large base deletions of 1 kb or more in the *CYBB* gene (four male patients, one female patient) and the mothers of four of those patients. The five Japanese patients were found to range from a patient exhibiting deletions only of the *CYBB* gene to a female patient exhibiting an extensive DNA deletion and the DMD and CGD phenotype manifested. Of the other three patients, two exhibited *CYBB*, *XK*, and *DYNLT3* gene deletions. The remaining patient exhibited both a deletion encompassing DNA subsequent to the *CYBB* region following intron 2 and the *DYNLT3* gene and a complex copy number variation involving the insertion of an inverted duplication of a region from the centromere side of *DYNLT3* into the deleted region.

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## Introduction

Chronic granulomatous disease (CGD [OMIM 306400]) is caused by a reduced nicotinamide adenine dinucleotide phosphate (NADPH) oxidase complex deficiency and is a primary immunodeficiency that impairs the bacteria-killing ability of phagocytes in the innate immune system and occurs with an incidence of 1 in 200,000 births per year [1]. The NADPH oxidase complex is localized in the cell membrane and is composed of heterodimeric membrane-bound flavocytochrome subunits formed from $gp91^{phox}$ (phox for phagocyte oxidase [OMIM 300481]) and $p22^{phox}$ (OMIM 233690) and cytoplasmic subunits formed from $p47^{phox}$ (OMIM 233700), $p67^{phox}$ (OMIM 233710), $p40^{phox}$, and *Rac2* (OMIM 602049). Reactive oxygen species (ROS) required to kill microorganisms, such as superoxide anions ($O_2^{\bullet -}$) and hydrogen peroxide, are produced by the transfer of electrons from NADPH to oxygen molecules. In CGD, however, since the NADPH oxidase complex is defective or dysfunctional, it is not possible to kill microorganisms, although the leukocyte phagocytoses the microorganisms, and this leads to repeated infections by fungi and catalase-positive bacteria, such as Staphylococcus, Pseudomonas aeruginosa, Escherichia coli, and Klebsiella pneumoniae, and frequently causes fatal granulomatous inflammation.

Mutations in genes for any of the six subunits of NADPH oxidase cause CGD, but almost 2/3 of CGD cases are caused by mutations in the X-linked *CYBB* gene, which codes for $gp91^{phox}$. Other CGD patients all show autosomal recessive inheritance patterns. *CYBB* is located at Xp21.1 and is a 33.4 kb gene made up of 13 coding exons and a promoter region mainly expressed in phagocytes. Its mutations have been reported to include non-sense mutations, missense mutations, splice site mutations, duplications, deletions, and insertions [2]. In Japan, more than 260 patients with CGD have been reported [3]. Genetic analysis of the 92 patients with *CYBB* gene mutations was performed. Mutations similar to those in the above reports were found, but in five of the 16 cases exhibiting deletions, the deletion was estimated to be large at 1 kb or more. Quite recently, details have been revealed from two patients of five exhibiting contiguous gene syndrome (CGS) associated with deletions of the *XK* gene responsible for McLeod syndrome (OMIM 314850) and the ornithine transcarbamylase (*OTC*) gene responsible for OTC deficiency (OTCD [OMIM 311250]) [4].

However, it is often difficult to detect multiple exon deletions and multigene deletions with conventional PCR-SSCP analysis and DNA sequencing, hindering the precise search for gene duplications and the detection of heterozygous deletions and duplications in female carriers. In addition, it is technically difficult to use Southern blot analysis for high precision, extensive deletion searches. Because of this, the details of the types of genomic rearrangements including duplications and deletions (genomic copy-number losses and gains) and their ranges (sizes and boundaries) in X-linked CGD caused by mutations in the *CYBB* gene are largely unresolved.

Array comparative genomic hybridization (aCGH) is a method that has been successfully adapted to the detection of copy number variations (CNVs) in OTCD and Duchenne/Becker muscular dystrophy (DMD [MIM 310200]), has been confirmed to show high sensitivity compared with traditional methods currently available, and has been developing rapidly in recent years [5,6]. In the present study, we analyzed genomic rearrangements in Japanese X-linked CGD patients using aCGH analysis, revealing the extent and the types of the deletion genes.

## Results

### Search for the *CYBB* gene and *XK* gene by PCR

In patients 1, 3, and 4, neither exon 1 nor exon 13 of the *CYBB* gene was detected. In patient 2, exon 1 of the *CYBB* gene was detected, but exon 13 was not. In patient 5, both exon 1 and exon 13 of the *CYBB* gene were detected. A search for the *XK* gene was performed. Exon 1 of the *XK* gene was detected in patients 1, 2, and 5, but not in patient 3 or 4. In the healthy controls, on the other hand, exons 1 and 13 of the *CYBB* gene and exon 1 of the *XK* gene were clearly detected (Figure 1). These results suggested that while genes are present upstream of the *XK* gene in patient 1, at the very least, all *CYBB* gene exons are deleted, and that extensive deletions encompassing the *CYBB* gene and *XK* gene are exhibited in patients 3 and 4. Deletion of *CYBB* exon 13 and the centromere side was suspected in patient 2. Patient 5 exhibited a similar gene amplification pattern to the healthy controls, and no obvious abnormality was observed.

### Deletion search by aCGH

The results of the search for CNVs by aCGH are summarized in Table 1. Deletions were observed in all five patients. Patient 1 showed the smallest deletion of the five patients at 58.7 kb, starting upstream of the *CYBB* gene and including all the exons of the 33.4 kb *CYBB* gene (Figure 2). Patient 2 was shown to exhibit a complex CNV involving a deletion of 84.4 kb encompassing the region downstream of exon 1 of the *CYBB* gene and the *DYNLT3* gene base sequence and a base sequence duplication of 91.9 kb adjacent to the centromere side of the deletion region (Figure 3). Patients 3 and 4 showed large deletions of 0.59 Mb and 1.94 Mb, respectively, encompassing the *XK* and *CYBB* genes and the *DYNLT3* gene (OMIM 300302), the function of which is not yet clear (Figure 4). Patient 5 showed an extensive deletion (5.71 Mb), exhibiting deletion of exons 1 to 42 of the 79 exons making up the *DMD* gene in addition to deletion of the *DYNLT3* gene, *CYBB* gene, and *XK* gene (Figure 5). aCGH of the mother of patient 5 also revealed a deletion of the same extent as in patient 5. The size of the genomic DNA deletions is half of that in the healthy female controls. Specifically, this shows that patient 5 has a deletion in the maternally inherited X-chromosome. With the exception of the mother of patient 4, who could not be tested, the mothers of the other four patients all had the same CNVs as their children, and the CNVs in patient 4 were heritable, rather than de novo (Table 1).

### Breakpoint analysis

A common TA base sequence was observed at breakpoints in patient 1 and the deletion of chrX:g.37364030_37428980del64950 (UCSC hg17 May 2004) was observed (Figure 2). The breakpoint at

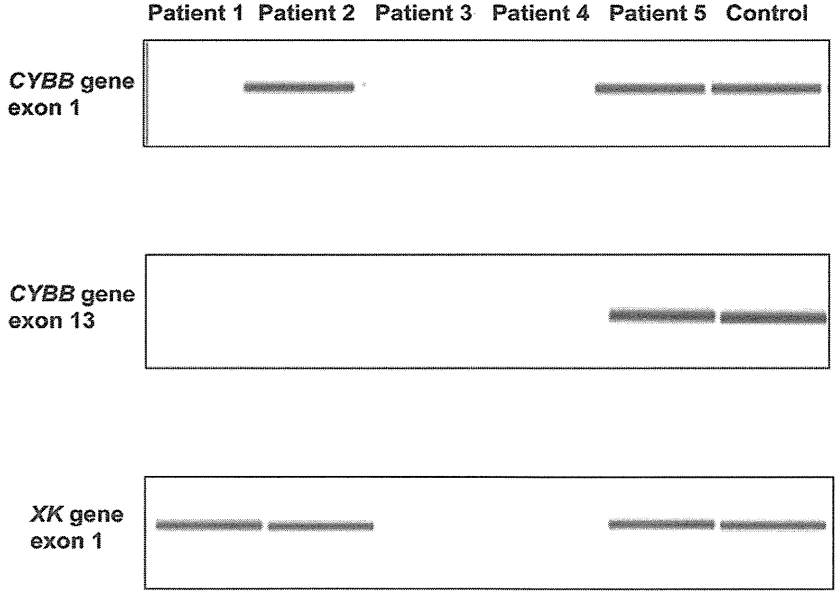


**Figure 1. PCR Amplification Results for Exons 1 and 13 of the Chronic Granulomatous Disease Gene *CYBB* and Exon 1 of the McLeod Syndrome Gene *XK* Region.** The analysis used the Agilent 2100 Bioanalyzer. Patients 1 to 5 were juvenile patients with chronic granulomatous disease, and genes from normal boys were amplified as a control. *CYBB* exon 1 was detected only from patients 2 and 5, and *CYBB* exon 13 only from patient 5. In addition, *XK* exon 1 was detected in patients 1, 2 and 5.
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