Table 2 (*continued*)

Approximate size of deletion (associated disease)	Affected exon(s)	CGD type	Acc. #	Ref	Kindred (patients)	
~3 kb	del exon 7	X91$^{?}$		Unpubl.	1(2)	*
~2.2 kb	del exon 5	X91°	A0206	[1,19]	1(1)	
~2 kb	del exon 3	X91°		[1]	1(1)	
~2 kb	del exon 7	X91°		[23]	1(1)	*
ND	del exon 3	X91$^{?}$		Unpubl.	1(1)	*
~2 kb	del promoter_exon 1	X91°		[1]	1(1)	
ND	del promoter_exon 1	X91$^{?}$		[23]	1(1)	*
~2 kb	del exon 8	X91°		[1] unpubl.	2(2)[a]	
2 kb	del exons 12_13	X91°		[1,17]	1(1)	
~2 kb	del. exon 7	X91°		[23]	1(1)	*
ND	del. exon 7	X91$^{?}$		Unpubl.	1(1)	*
~1.1 kb	del exon 6	X91$^{?}$		[23]	1(2)	*
~1 kb	del intron 12_ 3′UTR	X91°	A0203	[1,101] unpubl.	1(1)	
ND	del exon 9	X91$^{?}$		Unpubl.	1(1)	*
ND	del. exon 9	X91$^{?}$		Unpubl.	1(1)	*
0.35 kb	del exon 3	X91°		[1]	1(2)	
0.22 kb	del promoter	X91°		[1]	1(1)	

DMD, Duchenne muscular dystrophy; RP, X-linked retinitis pigmentosa; OTC, ornithine transcarbamylase deficiency; McLeod, McLeod hemolytic anemia; 3′UTR, 3′ untranslated region.

[a] These mutations are not necessarily identical.

[b] Patients A0038 and A0039 are brothers with different deletions.

[c] Patients A0027 and A0028 are brothers with different deletions; their mother has both mutations and the wild-type *CYBB* sequence (triple mosaic) [34,111].

Table 3
Known polymorphisms in the *CYBB* gene.

Nucleotide change	Effect	Approximate frequency
c.−270C/A	N.A.	Unknown [112]
c.141+48C/G	N.A.	Unknown (Maddalena, unpubl.)
c.142−12C/T	N.A.	Unknown (internet, unpubl.)
c.484−60delT	N.A.	Unknown (Jianxin He, unpubl.)
c.484−4G/A	Splice	Unknown [1]
c.654C/A	Silent (p.Gly218)	2% A in sub-Sahara Africans (internet, unpubl.)
c.804+118A/G	N.A.	Unknown (Maddalena, unpubl.)
c.1002G/A	Silent (p.Lys334)	4% A in sub-Sahara Africans (internet, unpubl.) [1]
c.1090G/C	p.364Gly/Arg	Unknown [1,113]
c.1414G/A	p.472Gly/Ser	2% A in Asians (internet, unpubl.)
c.1551T/A	p.517Asp/Glu	Unknown (Hill, unpubl.) [1]
c.1581C/T	Silent (p.His527)	Unknown (Di Matteo, unpubl.)

Table 4
Total number of kindreds with X-CGD patients, total number of X-CGD patients, total number of different mutations and total number of mutations unique for one kindred.

	Kindreds	Mutations
Deletions	281 (22.2%)	242 (35.6%)
Insertions	89 (7.0%)	54 (7.9%)
Deletion/insertions	19 (1.5%)	19 (2.8%)
Splice site mutations	247 (19.5%)	120 (17.6%) (2 undefined)
Missense mutations	246 (19.4%)	145 (21.3%)
Nonsense mutations	377 (29.8%)	96 (14.1%)
Promoter mutations	8 (0.6%)	5 (0.7%)
	Total 1267 unrelated kindreds with 1415 patients	Total 681 different mutations in the patients (all large deletions considered different). Of these 681 mutations, 498 (73.1%) are unique for one kindred.

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Brief report

Relapse of leukemia with loss of mismatched HLA resulting from uniparental disomy after haploidentical hematopoietic stem cell transplantation

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We investigated human leukocyte antigen (HLA) expression on leukemic cells derived from patients at diagnosis and relapse after hematopoietic stem cell transplantation (HSCT) using flow cytometry with locus-specific antibodies. Two of 3 patients who relapsed after HLA-haploidentical HSCT demonstrated loss of HLA alleles in leukemic cells at relapse; on the other hand, no loss of HLA alleles was seen in 6 patients who relapsed after HLA-identical HSCT. Single-nucleotide polymorphism array analyses of sorted leukemic cells further revealed the copy number-neutral loss of heterozygosity, namely, acquired uniparental disomy on the short arm of chromosome 6, resulting in the total loss of the mismatched HLA haplotype. These results suggest that the escape from immunosurveillance by the loss of mismatched HLA alleles may be a crucial mechanism of relapse after HLA-haploidentical HSCT. Accordingly, the status of mismatched HLA on relapsed leukemic cells should be checked before donor lymphocyte infusion. (*Blood.* 2010;115(15):3158-3161)

Introduction

Human leukocyte antigen (HLA) molecules expressed on the cell surface are required in presenting antigens to T cells. The HLA class I antigens are vital in the recognition of tumor cells by tumor-specific cytotoxic T cells. The loss of HLA class I molecules on the cell surface membrane may lead to escape from T-cell immunosurveillance and the relapse of leukemia. Previously, loss of HLA class I haplotype has been described in solid tumors.[1-3] However, there are few reports concerning HLA-haplotype loss in leukemia.[4,5]

We examined HLA class I expression in leukemic blasts from patients who relapsed after hematopoietic stem cell transplantation (HSCT) to analyze whether the loss of HLA on leukemic cells was related to the relapse after HLA-identical or haploidentical HSCT.

Methods

Patients and transplantation procedure

We identified 9 children with acute leukemia who relapsed after HSCT. Their leukemic samples were cryopreserved both at the time of the initial diagnosis and of relapse. The patients' characteristics are summarized in supplemental Table 1 (available on the *Blood* website; see the Supplemental Materials link at the top of the online article). Three patients received HSCT from an HLA-haploidentical family donor, and the other 6 patients received HSCT from an HLA-matched donor (4 siblings and 2 unrelated donors).

Written informed consent was given by the parents according to the protocol approved by the ethics committee of Nagoya University Graduate School of Medicine in accordance with the Declaration of Helsinki.

HLA class I expression on leukemic cells

Samples were collected at diagnosis and post-transplantation relapse. HLA expression of leukemic blasts and normal cells was analyzed by flow cytometry as previously reported.[6] Anti-HLA A2-FITC (cloneBB7.2) and anti-HLA A24-FITC (clone17a10) monoclonal antibodies were purchased from Medical & Biological Laboratories; HLA-A11 (IgM), HLA-A30, HLA-31 (IgM), HLA-25, HLA-26 (IgM), HLA-Bw6 (IgG3), and HLA-Bw4 (IgG3) antibodies were purchased from One Lambda. For leukemic cell markers, CD13-PE (IgG1) were purchased from Immunotech and CD34-APC (IgG1) were purchased from BD Biosciences. Samples were analyzed with FACSCalibur cytometer and CellQuest software. The method of genomic HLA typing was previously reported.[7]

Isolation of DNA and single nucleotide polymorphism analysis

The $CD13^+/CD34^+$ leukemic blasts were sorted by flow cytometry from bone marrow cells at the time of diagnosis and of relapse. Genomic DNA was extracted from leukemic cells sorted by a fluorescence-activated cell sorter as well as from phytohemagglutinin-stimulated patient-derived T cells and subjected to single nucleotide polymorphism (SNP) array analysis using GeneChip NspI arrays (Affymetrix) according to the manufacturer's protocol. Allele-specific copy number was detected using Copy Number Analyzer for GeneChip software as previously described.[8]

Limiting dilution-based CTLp frequency assay

The frequencies of cytotoxic T-lymphocyte precursor (CTLp) specific for the recipient-mismatched HLA molecules were analyzed using a standard limiting dilution assay.[9]

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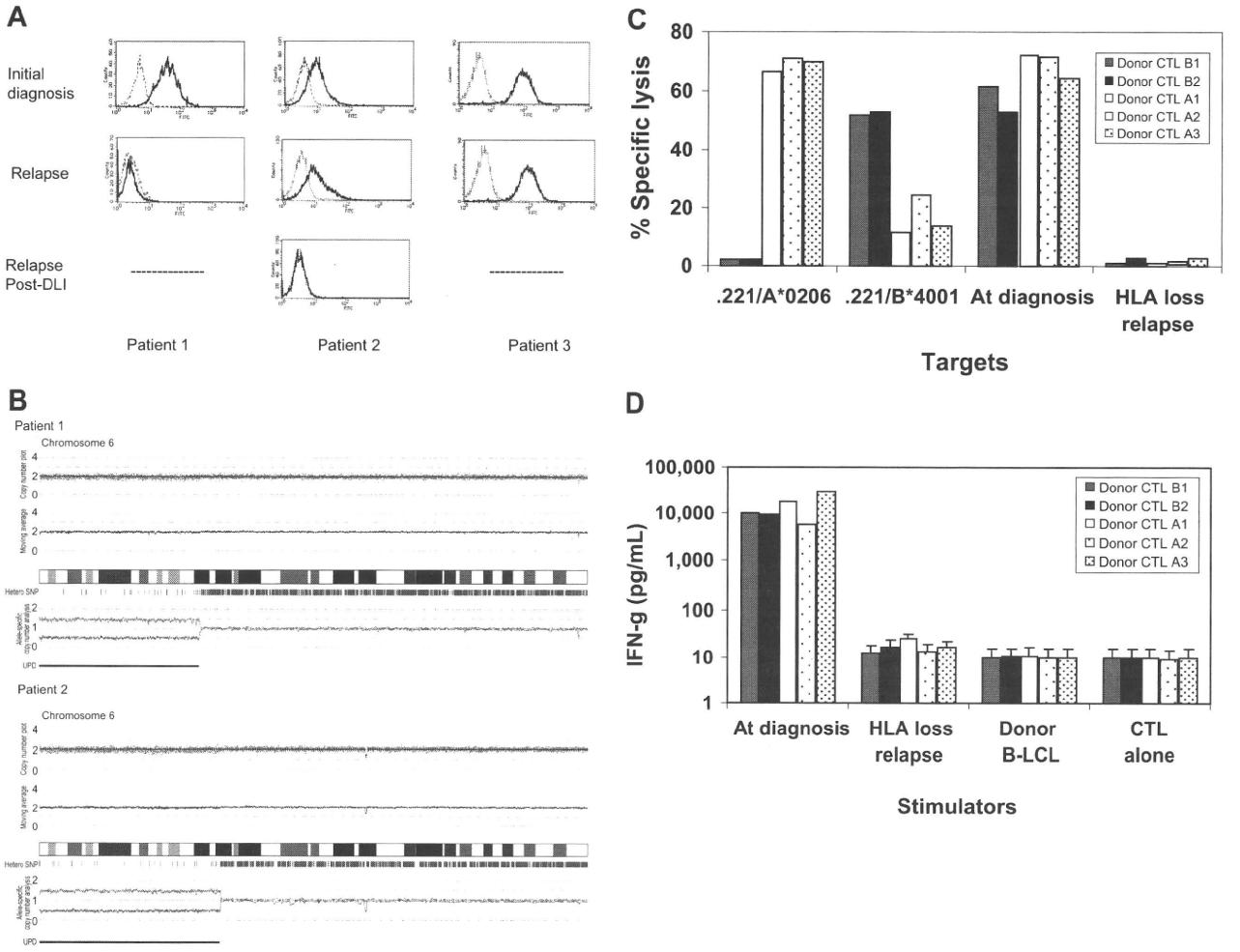

Figure 1. The loss of mismatched HLA expression on leukemic blasts caused by uniparental disomy on chromosome 6p impaired recognition and killing of donor's alloreactive cytotoxic T lymphocytes. (A) Leukemic blasts at the time of initial diagnosis and at the time of relapse after hematopoietic stem cell transplantation (HSCT) and donor lymphocyte infusion (DLI) were gated by $CD34^+$ and $CD13^+$, and then the surface expression of mismatched human leukocyte antigen (HLA) alleles was examined with anti–HLA-A2 antibodies. In 3 patients with acute myelogenous leukemia (AML) who experienced relapse after HLA-haploidentical HSCT, HLA-A2 expression was lost in patient 1 at relapse 15 months after HSCT and lost in patient 2 at second relapse 6 months after DLI. (B) Single nucleotide polymorphism (SNP) array analyses of sorted leukemic cells with the loss of an HLA allele revealed that the short arm of chromosome 6 shows copy number-neutral loss of heterozygosity or acquired uniparental disomy as detected by dissociated allele-specific copy number plots (red and blue lines at the bottom), resulting in the total loss of the mismatched HLA haplotype in both patient 1 and patient 2. The presence of acquired uniparental disomy is also indicated by normal total copy numbers with missing heterozygous SNPs (green bars) in the distal part of the short arm. (C) Recipient alloantigen-specific cytotoxic T-lymphocyte (CTL) clones were generated by a conventional cloning method from cytotoxicity-positive wells obtained in the limiting dilution assays using the donor $CD8^+$ cells as responders. Donor CTL clones A1, A2, and A3 were specific for HLA-A*0206. Donor CTL clones B1 and B3 were specific for HLA-B*4001, all of which recognize mismatched HLA alleles between the donor and recipient. Those 5 representative CTL clones were tested for HLA specificity and recognition of leukemic blasts obtained at the time of the initial diagnosis and at the time of HLA loss relapse after DLI by a standard ^{51}Cr-release assay at the effector/target ratio of 30:1. (D) Their interferon-γ production was also assessed against leukemic blasts collected at the time of diagnosis and at the time of HLA-loss relapse.

Cytotoxic assay of CTL clones against leukemic blasts and a mismatched HLA cDNA-transfected B-lymphoblastoid cell line

The remaining cells of several cytotoxicity-positive wells used for the CTLp assay for the donor were used to obtain allo-HLA–restricted CTLs. CTL clones were isolated by standard limiting dilution and expanded as previously described.[10,11]

The HLA class I–deficient 721.221 B-lymphoblastoid cell line was maintained in RPMI 1640 medium supplemented with 10% fetal bovine serum, 2mM L-glutamine, and 1mM sodium pyruvate. Retroviral transduction was conducted as previously described.[12]

The cytotoxicity of CTL clones against target cells was analyzed by conventional chromium 51 (^{51}Cr) release assay as previously reported.[13]

CTL clones (10^4 cells/well) were mixed with the indicated stimulator cells (10^4 cells/well) in 96-well, round-bottom polypropylene plates and spun at 1200*g* for 3 minutes before overnight incubation in 200 μL of RPMI 1640 medium supplemented with 10% fetal bovine serum. On the next day, 50 μL of supernatant was collected and interferon-γ was measured by enzyme-linked immunosorbent assay with 3,3′,5,5′-tetramethylbenzidine substrate (Sigma-Aldrich).

Results and discussion

Three children with high-risk acute myelogenous leukemia (AML) received haploidentical grafts from their parents but relapsed 8, 14, and 15 months after HSCT. Patient 2 received 3 courses of donor lymphocyte infusion (DLI) for relapsed leukemia after haploidentical HSCT. After the third unmanipulated DLI (10^7 $CD3^+$/kg), she experienced acute grade-III graft-versus-host disease and achieved complete remission. However, she experienced a second relapse 6 months later. To monitor residual disease in those patients, we used flow cytometric analysis with antibodies specific for the mismatched HLA alleles between the donor and patient. Surprisingly, we found total loss of

HLA-A2 expression on $CD13^+/CD34^+$ leukemic cells from bone marrow in 2 of 3 patients who underwent HLA-haploidentical HSCT, whereas microscopic analysis showed relapse (Figure 1A). To test whether HLA class I molecules could be up-regulated, samples were cultured for 48 hours in medium supplemented with tumor necrosis factor-α or interferon-γ and measured again; however, no restoration was observed (data not shown).

Next, to examine the potential loss of genes encoding the undetectable HLA alleles, we sorted $CD13^+/CD34^+$ leukemic blasts and performed DNA genotyping. We found that, in addition to the HLA-A locus, the HLA-B, -C, and -DR loci were not encoded; only the mismatched haplotype was lost in both patients (supplemental Table 2). We then questioned whether this phenomenon would also occur in HLA-matched HSCT settings using anti-HLA class I antibodies. We did not observe any loss of HLA class I expression in any of the patients at the time of relapse (supplemental Figure 1). These results suggest that loss of HLA class I haplotype at the time of posttransplantation relapse is uncommon in HLA-matched HSCT.

To elucidate the mechanism of the loss of the mismatched HLA haplotype, we performed an SNP array analysis of genomic DNA extracted from leukemic blasts at the time of diagnosis and of relapse. Genomic DNA from patient-derived T cells was used as a reference. Leukemic cells at the time of relapse showed copy number-neutral loss of heterozygosity or an acquired uniparental disomy (UPD) of the short arm of chromosome 6 encompassing the HLA locus, whereas no allelic imbalance was identified at the time of diagnosis (Figure 1B). Loss of one allele from one parent and duplication of the remaining allele from the other parent led to UPD.[14]

In patient 2, we examined whether the number of CTLp had changed during the posttransplantation course. Limiting dilution analysis with a split-well ^{51}Cr-release assay was carried out to compare the CTLp frequencies specific for the mismatched antigens between the recipient and donor. Interestingly, the CTLp frequencies were recovered after DLI (Table 1). Restoration of CTLp after 3 DLIs could eradicate such leukemic cells, lasting for 6 months thereafter.

Next, we generated allo-HLA–restricted CTLs from $CD8^+$ cells obtained at day 520 in patient 2 and tested with the 721.221 B-lymphoblastoid cell line transfected with 1 of 3 mismatched HLA alleles (Figure 1C-D).

Despite high transplantation-related mortality resulting from severe graft-versus-host disease and posttransplantation infections, haploidentical HSCT has been widely used with the expectation of a strong graft-versus-leukemia effect.[15] However, our observation provides a possible limitation of this strategy. Indeed, 2 of 3 patients showed genomic loss of the recipient-specific HLA-haplotype, which led to escape from the graft-versus-leukemia effect and relapse of the disease.

Vago et al also reported a similar observation in 5 of 17 (29.4%) patients whose disease relapsed after haploidentical HSCT.[16] Relapsed leukemic cells may possess genomic instability that elicits genetic diversity.[17] Immunologic pressure by alloreaction to major HLA antigens may select leukemic variants of HLA class I loss, which results in the survival and proliferation of these variants.

In haploidentical HSCT, the importance of natural killer (NK)–cell alloreactivity is emphasized to achieve the graft-versus-leukemia effect.[18,19] HLA loss on leukemic blasts may in turn enhance the NK-cell alloreactivity. Our 2 patients with HLA loss had a group 1 homozygous HLA-C locus that is a suppressive killer immunoglobulin-like receptor (KIR) for NK cells and a KIR-matched donor (supplemental Table 2). Because UPD does not change the total copy number of the gene, donor NK cells should have been suppressed even after UPD occurred in these patients. Interestingly, the remaining patient who experienced relapse without HLA loss after HLA-haploidentical HSCT had a KIR-mismatched donor, so alloreactive NK cells were possibly enhanced to kill leukemic blasts with HLA loss.

Although one limitation of our study is an insufficient number of cases, our results combined with those in a recent report[16] suggest that leukemic cells occasionally escape from immunosurveillance through the loss of the mismatched HLA haplotype by the mechanism of UPD after haploidentical HSCT. DLI for relapsed AML is less effective than that for chronic myelogenous leukemia after HLA-matched HSCT.[20] However, DLI is effective even for the relapse of AML after haploidentical HSCT.[21] Evaluation of loss or down-regulation of HLA on relapsed leukemic blasts after HLA-haploidentical HSCT should be considered because DLI would probably be ineffective in patients whose leukemic cells lose HLA class I antigen.

Table 1. The CTLp frequency reactive to the recipient alloantigen in the recipient after transplantation and the donor

Samples	Maximum $CD8^+$ input*	No. of growing wells†	CTLp frequency^{-1} (95% confidence interval)
Donor	33 300	8	8.6×10^5 (1.49×10^6-5.0×10^5)
Day 100	35 500	0	UD
Day 180	17 700	0	UD
Day 300‡	86 000	0	UD
Day 520§	95 000	7	4.3×10^5 (7.2×10^5-2.5×10^5)

Purified $CD8^+$ T cells from the peripheral blood mononuclear cells obtained after transplantation from patient 2 and her donor were cultured at 2- or 3-fold serial dilutions with 33 Gy-irradiated 3×10^4 leukemic blasts cryopreserved at the time of initial diagnosis in 96-well, round-bottom plates in advanced RPMI 1640 medium supplemented with 4% pooled human serum, interleukin-6 (IL-6), and IL-7 (10 ng/mL; both from R&D Systems). The IL-2 (50 U/mL) was added on day 7 with a half medium change. For each dilution, there were at least 12 replicates. On day 14 of culture, a split-well analysis was performed for recipient-specific cytotoxicity against ^{51}Cr-radiolabeled recipient T-cell blasts, donor T-cell blasts, and leukemic blasts harvested at the time of initial diagnosis and at the time of relapse after DLI if indicated. The supernatants were measured in a γ counter after 4-hour incubation. The wells were considered to be positive for cytolytic activity if the total counts per minute released by effector cells was more than 3 SD above the control wells (mean counts per minute released by the target cells incubated with irradiated stimulator cells alone). The CTLp frequency was calculated using L-Calc software (StemCell Technologies). The CTLp frequencies reactive with recipient T-cell blasts in $CD8^+$ T cells obtained around days 100, 180, and 300 (4 months before relapse) were undetectable, whereas the CTLp frequency obtained at day 520 (1 month after the third DLI or 2 weeks after remission confirmed by bone marrow aspirate) was close to the CTLp frequency in the donor $CD8^+$ cells. Complete remission and more than 99% donor chimerism were confirmed on those days

CTLp indicates CTL precursor; and UD, undetermined because no growing wells are present.

*Number of input $CD8^+$ T cells seeded at the highest number per well.

†Number of wells out of 12 wells that received the highest $CD8^+$ cells and showed detectable growth.

‡Corresponds to 4 months before relapse.

§Corresponds to 1 month after the third DLI or 2 weeks after complete remission was confirmed by bone marrow aspirate.

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Authorship

Contribution: I.B.V. performed experiments and wrote the manuscript; Y.T. designed the research, analyzed data, and wrote the manuscript; Y.A., H.S., M.K., and S.O. performed experiments, analyzed data, and wrote the manuscript; S.K. supervised this work and wrote the manuscript; and all other authors were responsible for clinical work and critically reviewed the manuscript.

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Mutations of an E3 ubiquitin ligase *c-Cbl* but not *TET2* mutations are pathogenic in juvenile myelomonocytic leukemia

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Juvenile myelomonocytic leukemia (JMML) is a rare pediatric myeloid neoplasm characterized by excessive proliferation of myelomonocytic cells. When we investigated the presence of recurrent molecular lesions in a cohort of 49 children with JMML, neurofibromatosis phenotype (and thereby *NF1* mutation) was present in 2 patients (4%), whereas previously described *PTPN11*, *NRAS*, and *KRAS* mutations were found in 53%, 4%, and 2% of cases, respectively. Consequently, a significant proportion of JMML patients without identifiable pathogenesis prompted our search for other molecular defects. When we applied single nucleotide polymorphism arrays to JMML patients, somatic uniparental disomy 11q was detected in 4 of 49 patients; all of these cases harbored RING finger domain *c-Cbl* mutations. In total, *c-Cbl* mutations were detected in 5 (10%) of 49 patients. No mutations were identified in *Cbl-b* and *TET2*. *c-Cbl* and *RAS* pathway mutations were mutually exclusive. Comparison of clinical phenotypes showed earlier presentation and lower hemoglobin F levels in patients with *c-Cbl* mutations. Our results indicate that mutations in *c-Cbl* may represent key molecular lesions in JMML patients without *RAS*/*PTPN11* lesions, suggesting analogous pathogenesis to those observed in chronic myelomonocytic leukemia (CMML) patients. (Blood. 2010;115:1969-1975)

Introduction

Juvenile myelomonocytic leukemia (JMML) is a special subtype of myelodysplastic syndrome/myeloproliferative disorder (MDS/MPD) that, analogous to chronic myelomonocytic leukemia (CMML), is characterized by excessive proliferation of myelomonocytic cells, but unlike CMML it occurs in young children and shows characteristic hypersensitivity to granulocyte-macrophage colony-stimulating factor (GM-CSF).[1-3] Mutations of genes involved in GM-CSF signal transduction, including *RAS* and *PTPN11*, can be identified in a majority of children with JMML.[3-5] Constitutional mutations of *NF1* can be found in another 10% of patients with JMML.[1,6,7] Recent studies show that a common mechanism of *NF1* inactivation is uniparental disomy (UPD) resulting in duplication of the mutant *NF1* allele.[7,8] *NF1* is a GTPase activating protein for RAS and thereby acts as a tumor suppressor.[9] Oncogenic *RAS* mutations at codons 12, 13, and 61 have been identified in approximately 20% to 25% of patients with JMML.[4,10] These mutations lead to elevated levels of RAS-GTP, the active form of RAS.[11] Somatic mutations in *PTPN11*, coding for tyrosine phosphatase Src homology 2 domain-containing protein, have been reported in 35% of patients with JMML,[5,12,13] and induce hematopoietic progenitor hypersensitivity to GM-CSF due to hyperactivation of the RAS signaling axis.[14,15]

Based on the proposed paradigm that recurrent areas of somatic copy-neutral loss of heterozygosity can point toward the presence of homozygous mutations contained within the corresponding region,[16] we have identified various recurrent areas of acquired segmental UPD, in particular in patients with MDS/MPD, including CMML. Such analyses have shown that, in addition to the recently identified *Jak2V617F* mutation associated with UPD9p, other known mutations can be duplicated by homologous recombination, including, for example, *c-Mpl* (UPD1p), *FLT-3* ITD (UPD13q), *TET2* (UPD4q), and others.[17-22] Based on the observation of recurrent somatic UPD11q23.3, we have discovered homozygous *c-Cbl* mutations in the RING finger domain (RFD) occurring frequently in MDS/MPD and especially CMML or secondary acute myeloid leukemia (AML) evolved from CMML.[23] When we analyzed other members of *Cbl* gene family, mutations were also found in *Cbl-b* and *Cbl-c* and were associated with an indistinguishable clinical phenotype.[24] The *Cbl* gene family codes for E3 ubiquitin ligases (ULs) with the ubiquitination activity mediated via the RFD. They are involved in degradation of activated phosphotyrosine receptors and other phosphotyrosine kinases such as ζ-chain–associated protein kinase 70 involved in signal transduction.[25] Thus, mutations in the RFD can lead to decreased receptor degradation and, analogous to *PTPN11* mutations, result in augmentation of proliferative signals mediated by various growth factor receptors. In a *c-Cbl*$^{-/-}$ mouse model a mild myeloproliferative phenotype with expansion of stem cells and hyperresponsiveness to growth factors is found,[26] whereas a RFD mutant knock-in model shows a severe myeloproliferative phenotype (W. Langdon, University of Western Australia, oral communication, January 2009). These observations, together with the transforming effects of the

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v-Cbl oncogene lacking the RFD, suggest that E3 UL activity is essential for the tumor suppressor function of *c-Cbl,* whereas the N-terminal portion of the protein may be oncogenic.

Based on the morphologic similarities of JMML and typical CMML, presence of growth factor hypersensitivity, and observation of UPD11q in children affected by JMML, we hypothesized that *Cbl* family mutations may also be present in a subset of patients with JMML. Here, we investigated 49 JMML patients with the goals of (1) identifying pathogenic molecular lesions, including mutations in *Cbl* gene family members, and (2) correlating clinical outcomes to presence and location of other pathogenic molecular lesions, including *PTPN11*, *NRAS*, *KRAS*, and *TET2*. Of note is that during review of our paper, *c-Cbl* mutations were reported in JMML.[27]

Methods

Patients

We studied 49 children (32 boys and 16 girls; 1 patient's sex was unknown) with JMML diagnosed between 1988 and 2008 in 28 institutions throughout Japan. Written informed consent for sample collection was obtained at appropriate institutions from patients' parents according to the institutional protocols and the Declaration of Helsinki. The sample repository was located at Nagoya University Graduate School of Medicine. Molecular analysis of the mutational status was approved by the Ethics Committee of Nagoya University Graduate School of Medicine. The diagnosis of JMML was based on the internationally accepted criteria previously published.[26] We excluded patients with Noonan syndrome. The clinical and hematologic characteristics of the patients are summarized in Table 1. The median age at diagnosis was 28 months (range, 1-75 months). Karyotypic abnormalities were detected in 11 patients, including 7 patients with monosomy 7. Two children had clinical evidence of *NF1*. Of 49 patients, 32 underwent hematopoietic stem cell transplantation.

Table 1. Characteristics of JMML patient cohort

Variable	Total cohort, N = 49
Median age at diagnosis, mo (range)	32 (1-75)
Sex, male/female/unknown	32/16/1
NF1 by clinical diagnosis, yes/no	2/47
Median Hb, g/L (range)	0.96 (0.49-1.20)
Median HbF, % (range)	23.6 (1.0-62.0)
Median WBC, $\times 10^9$/L (range)	28.0 (10.9-126.2)
Median monocyte in PB count, $\times 10^9$/L (range)	4.5 (1.0-31.6)
Median plt, $\times 10^9$/L (range)	49 (1.4-320)
Metaphase cytogenetics, no. of patients (%)	
Normal karyotype	35 (71.4)
Monosomy 7	8 (16.3)
Trisomy 8	1 (2.0)
Other abnormalities	3 (6.1)
Unknown	2 (4.1)
Hematopoietic stem cell transplantation	
Yes	32
No	13
Unknown	4
Status at last follow-up	
Alive	24
Dead	21
Unknown	4
Median observation period, mo (range)	14 (1-216)

NF1 indicates neurofibromatosis type1; Hb, hemoglobin; WBC, white blood cell; PB, peripheral blood; and plt, platelet.

SNP-A karyotyping analysis

Mononuclear cells were isolated using Ficoll-Hypaque density gradient centrifugation and cryopreserved until use. Genomic DNA was extracted using the QIAamp DNA Blood Mini Kit (QIAGEN). High-density Affymetrix single nucleotide polymorphism array (SNP-A; 250 K) was applied as a karyotyping platform to identify loss of heterozygosity (LOH), microamplification, and microdeletion as previously described.[28]

Bioinformatic analysis

Signal intensity was analyzed and SNP calls were determined using Gene Chip Genotyping Analysis Software Version 4.0 (GTYPE). Copy number and areas of UPD were investigated using a Hidden Markov Model and CN Analyzer for Affymetrix GeneChip Mapping 250-K arrays (CNAG Version 3.0) as previously described.[28]

We excluded germline-encoded copy number variation and nonclonal areas of gene copy number–neutral LOH from further analysis using a bioanalytic algorithm based on lesions identified by SNP-A in an internal control series (N = 713) and reported in the Database of Genomic Variants (http://projects.tcag.ca/variation).[29] Through calculation of their average sizes, we defined a maximal size of germline LOH in controls and consequently excluded all defects of this type in patients' samples; according to 95% confidence interval, stretches of UPD larger than 25.8 Mb were considered unlikely of germline origin. In addition, all nonclonal areas of UPD seen in controls were interstitial.

PTPN11, *NRAS*, *KRAS*, *TET2*, and *E3* ubiquitin ligase mutational screening

To screen for *PTPN11* mutations, we polymerase chain reaction amplified genomic DNA corresponding to exons 2, 3, 4, 7, 8, 12, and 13 as previously reported.[12,30,31] *NRAS* and *KRAS* mutations in codons 12, 13, and 61 were identified as previously described[32,33] and were confirmed by sequencing. To screen patients for mutations in E3 ubiquitin ligase genes and *TET2*, direct genomic sequencing of exons constituting the RFD of *Cbl* family members (exons 8 and 9 of *c-Cbl,* exons 9 and 10 of *Cbl-b,* exons 7 and 8 of *Cbl-c,* and exons 3-11 of *TET2*) was performed. For sequencing, 250 ng of polymerase chain reaction product, 3μM original forward or reverse primer, 2 μL of Big Dye Version 3.1 (Applied Biosystems), and 14.5 μL of deionized H_2O were amplified under the following conditions: 95°C (2 minutes) followed by 25 cycles of 95°C (10 seconds), 50°C (5 seconds), and 60°C (4 minutes). Sequencing was performed as previously described.[22]

GM-CSF hypersensitivity assay

GM-CSF hypersensitivity assays were established as described previously.[2] Briefly, we used cytokine-free methocult H4230 (StemCell Technologies), and added 1×10^3 $CD34^+$ bone marrow cells that were prepared by positive selection with magnetic-activated cell sorting beads (Miltenyi Biotec). Recombinant human GM-CSF (R&D Systems) was added at the time the cultures were initiated. Cultures were performed in duplicate, and colonies of 40 or more cells were scored after 14 days of incubation. The data are expressed as percentage of maximal numbers of granulocyte-macrophage colony-forming units (CFU-GMs). This approach more accurately reflects changes in sensitivity and does not bias the results compared with graphing actual counts because most JMML samples had considerably higher total numbers of CFU-GMs than controls, although there was considerable patient-to-patient variability.

Statistical analysis

When appropriate, Kaplan-Meier statistics were applied to assess survival. For comparison of the frequency of mutation or other clinical features between disease groups, categoric variables were analyzed using the Fisher exact test and continuous variables were tested using the Mann-Whitney *U* test.

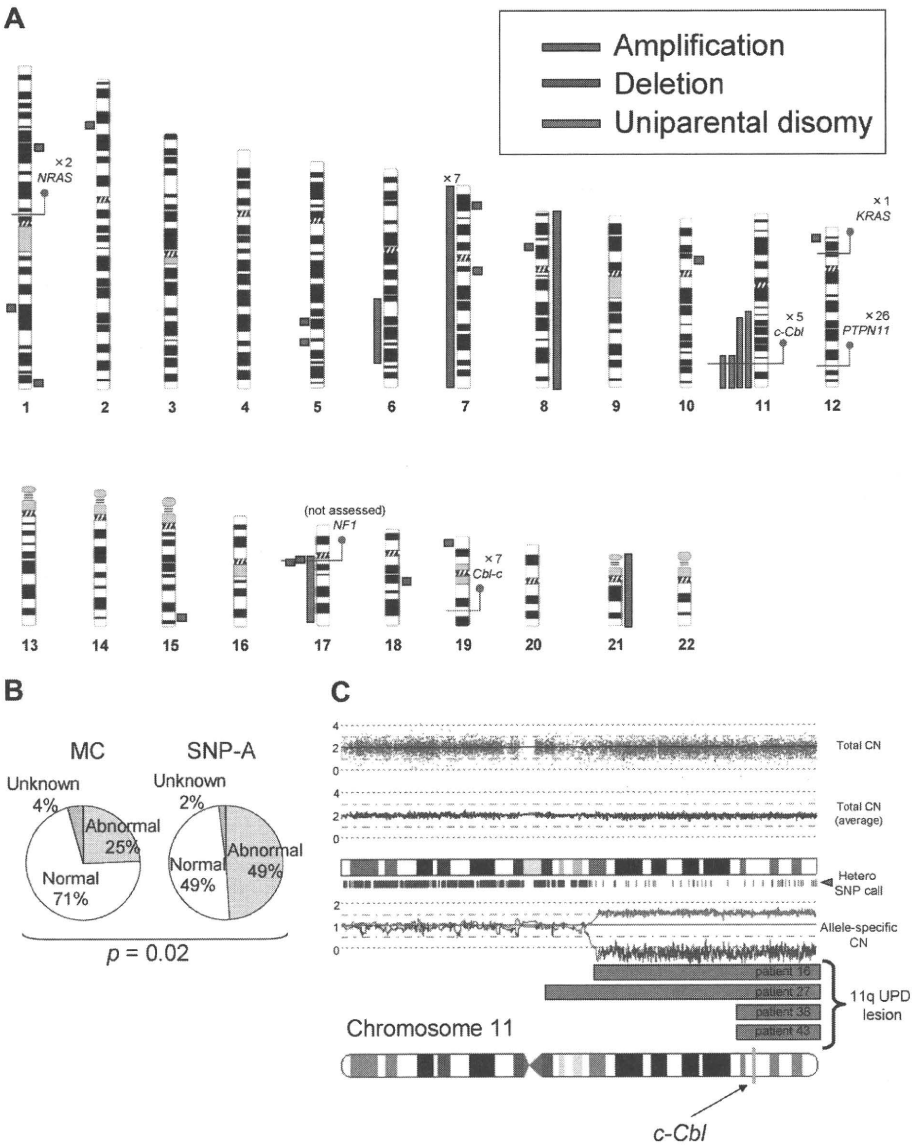


Figure 1. Single nucleotide polymorphism array–based karyotyping of JMML. (A) Genomic distribution and type of lesion identified in patients with JMML by SNP-A analysis. Green bar represent amplification, red shows deletion, and blue corresponds to UPD. Red lines pinpoint the locus of genes discussed in the text, as well as the number of patients mutated at that locus. *NF1* mutational status was not assessed in this cohort. (B) Increased sensitivity of SNP-A for detecting chromosomal lesions. The results of MC (25%) and by SNP-A (49%) from the JMML cohort studied are shown. (C) Representative 250-K SNP-A analysis of UPD11q by CNAG Version 3.0 (patient 16). Both the raw and averaged total copy number (CN) tracks (red dots, blue line) show a normal copy number, whereas heterozygous SNP calls and allele-specific copy number tracks (green dashes, red/green lines) show a reduction in copy number, indicating UPD. The specific localization of 11qUPD in 4 patients (patients 16, 27, 38, and 43) is indicated by the blue bars. The *c-Cbl* locus is indicated on the chromosome 11 idiogram with a yellow line.

Results

Cytogenetic and clinical characterization of JMML patients

First, we performed SNP-A– and metaphase cytogenetics–based analyses. Using conventional metaphase cytogenetics, chromosomal aberrations were found only in a minority of patients (25%). SNP-A–based karyotyping confirmed the results of metaphase cytogenetics, including the presence of monosomy 7 in 7 patients and trisomy 8 in 1 patient. However, due to increased precision and ability to detect copy-neutral loss of heterozygosity of SNP-A, additional lesions were identified by SNP-A in 24 (49%) of 48 patients, including trisomy 21 not detected by metaphase cytogenetics (MC) in 1 patient and microdeletions in 9 patients (Figure 1A), including 1q25.3 (patient 44), 2p22.1 (patient 46), 5q23.1 (patient 20), 5q31.3 (patient 40), 6q21q25.3 (patient 15), 8p21.2 (patient 36), 12p13.2 (patient 3), 17q11.2 (patients 15, 49), and 19p13.3 (patient 2). We also detected microamplifications in 7 patients (Figure 1A), located at 1p31.1 (patient 14), 1q44 (patient 39), 7p21.1 (patient 16), 7q11.22 (patient 17), 10p11.23 (patient 29), 15q26.3 (patient 47), and 18q12.3 (patient 22). The shared copy number–altering lesions included monosomy 7 and loss of 17q11.2, which contained the *NF1* locus. Although we were unable to confirm the somatic nature of the submicroscopic defects due to lack of germline DNA, these lesions did not overlap with copy number variations present in internal control cohort and publicly available databases. Most significantly, we identified UPD in 5 patients (Figure 1A). UPD11q was found in 4 patients, all regions overlapping from 11q23.3 to the telomere. This commonly affected region contained the *c-Cbl* locus (Figure 1A,C). The region of UPD at 17q contained the *NF1* locus and corresponded with clinical neurofibromatosis features. Overall, compared with the results of MC, SNP-A identified significantly more genetic abnormalities (25% vs 49%; $P = .02$; Figure 1B).

Table 2. Patients' mutational status

Patient number	*PTPN11*	*NRAS*	*KRAS*	*c-Cbl*	*Cbl-b*	*TET2*
1	■					
2	■					
3						
4						
5						
6						
7	■					
8	■					
9	■					
10	■					
11						
12						
13	■					
14	■					
15						
16				■		
17	■					
18	■					
19						
20						
21	■					
22	■					
23				■		
24	■					
25			■			
26		■				
27				■		
28	■					
29	■					
30		■				
31	■					
32	■					
33						
34						
35						
36	■					
37	■					
38				■		
39	■					
40	■					
41	■					
42	■					
43				■		
44						
45	■					
46						
47	■					
48						
49	■					
Total	**26**	**2**	**1**	**5**	**0**	**0**

Gray cells represent mutation; and white cells, wild type.

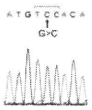
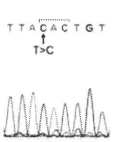
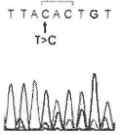
Mutational analysis of patients with JMML

After defining chromosomal defects associated with JMML, we performed mutational analysis of the genes known to be affected by mutations in JMML. *PTPN11* mutations were found in 26 (53%) of 49, whereas *NRAS* and *KRAS* mutations were found in 2 (4%) of 49 and 1 (2%) of 49, respectively (Table 2). None of the patients screened show the presence of *TET2* mutations, previously shown to be present in a significant proportion of patients with MDS/MPD, including CMML.[21] Excluding patients with a neurofibromatosis phenotype, 18 (37%) of 49 of patients did not show any of the known pathogenic defects occurring in JMML.

Identification of *Cbl* gene family mutations in JMML

Previously, homozygous *c-Cbl* mutations in the RFD were identified in patients with MDS/MPD, especially CMML or secondary AML that evolved from CMML.[22] We focused our attention on this gene as UPD11q was found in 4 of 49 JMML patients. Mutational analysis of *Cbl* family genes revealed mutations of *c-Cbl* in 5 (10%) of 49 patients, and no *Cbl-b* mutations (Tables 2-3). *c-Cbl* mutations were heterozygous in 1 patient (patient 23) and homozygous in 4 patients (patients 16, 27, 38, 43; supplemental Table 1, available on the *Blood* website; see the Supplemental Materials link at the top of the online article). All mutations were located in the RFD (exon 8 and intron 8); 2 patients had an identical homozygous mutation (1111T>C, Tyr371His; patients 27, 38). All 4 patients with a homozygous *c-Cbl* mutation simultaneously harbored UPD11q (supplemental Table 1, Figures 1C-2). In addition, no patient with a *c-Cbl* mutation had mutations in genes known to play a role in JMML (*PTPN11*, *NRAS*, and *KRAS*; Table 2) or had clinical diagnosis of NF1. Excluding patients with a neurofibromatosis phenotype, 13 (26.5%) of 49 of patients did not have the mutation of *PTPN11*, *RAS*, and *c-Cbl* genes.

Table 3. Summary of *c-Cbl* mutations in patients with JMML

Patient number	Pherograms of sequence	*c-Cbl* mutation			
		Homo /Hetero	Nucleotide change	Amino acid change	Location
16		Homo	1202 G>C	Cys401Ser	Exon 8
23		Hetero	1106 del 66bp	Deletion	Exon 8
27		Homo	1111 T>C	Tyr371His	Exon 8
38		Homo	1111 T>C	Tyr371His	Exon 8
43		Homo	1228-2 A>G	Splice acceptor site	Intron 8

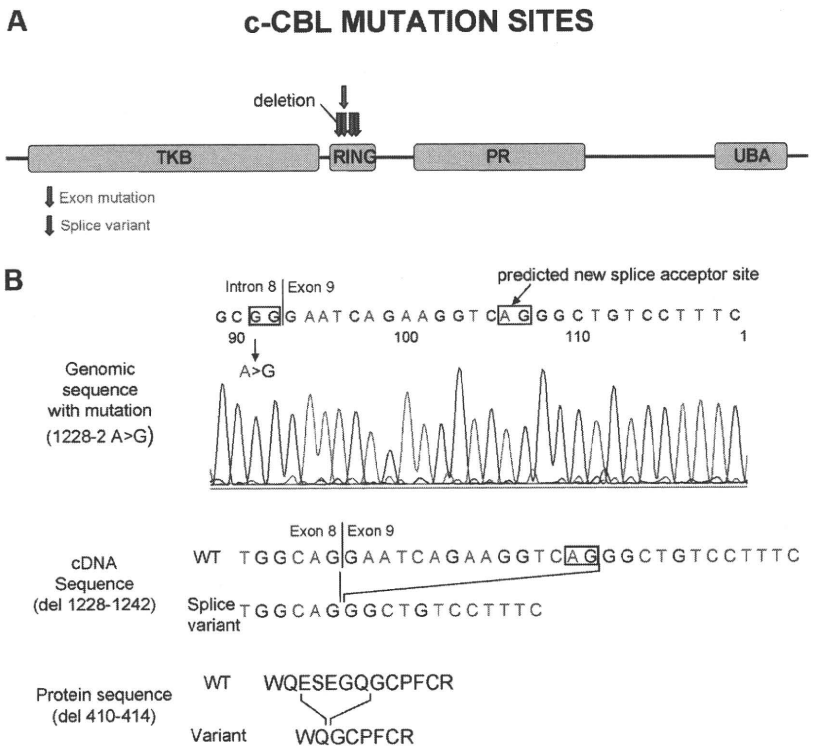


Figure 2. Site of the *c-Cbl* mutations and predicted product of splice variant in the intron 8 splice acceptor site. (A) Localization of the c-Cbl mutations within the predicted protein product. Red arrows show the site of mutations in exon, and blue arrows show the site of splice variant. (B) In patient 48, a homozygous mutation was seen in the intron 8 splice acceptor site of *c-Cbl*. According to http://genome.cbs.dtu.dk/services/NetGene2,[34] this mutation may result in a splice variant, leading to a shorter transcript in RF domain.

When we investigated our cohort for the presence of *Cbl-c* mutations, we found heterozygous frameshift nucleotide variation (1256 insertion C; patients 7, 12, 14, 29, 33, 38, and 46; data not shown). However, *Cbl-c* mutational status of germline though sequencing of nonclonal $CD3^+$ lymphocytes in those patients showed the same frameshift mutation. Consequently, these *Cbl-c* nucleotide exchanges represent rare polymorphisms.

GM-CSF hypersensitivity assay

We also investigated whether JMML-specific GM-CSF is related exclusively to individual types of mutations identified, including c-Cbl mutations. $CD34^+$ bone marrow cells' colony counts are expressed as percentage of maximal (supraoptimal) number of CFU-GMs (colony counts at any given concentration of GM-CSF/colony counts at 10 ng/mL GM-CSF). The colony growth of JMML cells with or without *c-Cbl* mutation did not differ from normal controls in low concentration of GM-CSF. For example at 0.01 ng/mL GM-CSF, colony counts were 55% (± 8%) with *c-Cbl* mutation (n = 4) versus 65% (± 10%) without *c-Cbl* mutation (n = 14) versus 15% (± 5%) in controls (n = 2; $P = .042$). At 0.1 ng/mL GM-CSF, colony counts were 87% (± 6%) versus 83% (± 11%) versus 15% (± 5%; $P = .011$) and at 1.0 ng/mL GM-CSF, 94% (± 11%) versus 93% (± 7%) versus 43% (± 3%; $P = .063$), respectively. Consequently, our results indicate that GM-CSF hypersensitivity of $CD34^+$ cells from JMML patients may be a result of various molecular lesions including *c-Cbl* mutations (Figure 3).

Clinical features associated with *Cbl* gene family mutations

Although different molecular lesions can result in similar clinical phenotypes, specific mutations can modify clinical behavior and morphologic features. Consequently, we analyzed clinical characteristics of patients with specific mutations (Table 4).

We did not find any distinctive morphologic features of patients with *Cbl* gene family mutations and no differences were present in the blood counts at initial presentation. Other variables studied (sex, the presence of cytogenetic abnormalities) also did not differ between patients grouped according to mutational status. However, patients with mutant *c-Cbl* compared with those with wild-type constellation showed earlier presentation (median age at diagnosis, 12 months vs 29 months, $P = .037$) and lower median hemoglobin F (HbF) percentage (3.5% vs 24.9%, $P = .02$), previously shown to correlate with less favorable prognosis.[1,33,35-40] Low HbF values in *c-Cbl* mutant cases were not attributable to monosomy 7, absent in this patient cohort. The probability of 2-year overall survival of *c-Cbl* mutant patients (50.0%; 95% confidence interval [CI], 25.0%-75.0%; n = 4) was similar to that of patients without *c-Cbl* mutations (50.4% [95% CI, 42%-59%]; n = 41). Similarly, when

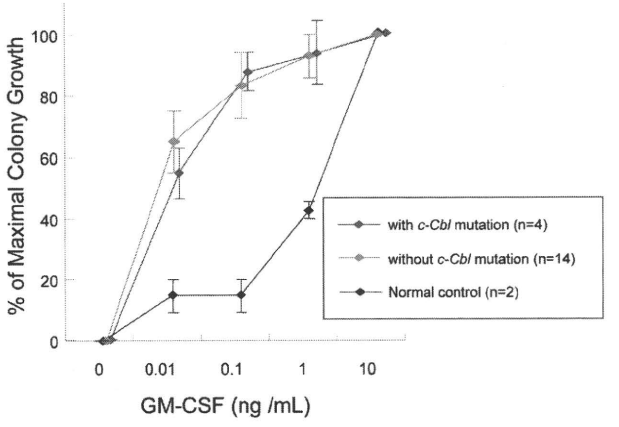


Figure 3. GM-CSF hypersensitivity assay. Colony counts are expressed as percentage of maximal numbers of CFU-GM (colony counts cultured with each concentration of GM-CSF/colony counts cultured with 10 ng/mL GM-CSF). The similar GM-CSF hypersensitivity was seen in JMML patients with or without *c-Cbl* mutation. Error bars represent SE.

Table 4. Comparison of clinical characteristics for JMML patients with and without *c-Cbl* mutation

Variable	With *c-Cbl* mutation, n = 5	Without *c-Cbl* mutation, n = 44	*P*
Median age at diagnosis, mo (range)	12 (8-15)	29 (1-75)	.037
Median HbF, % (range)	3.5 (2.0-7.6)	24.9 (1.0-62.0)	.02

Other variables studied (sex, hemoglobin level, white blood cell count, platelet count, monocyte percentage in peripheral blood, and metaphase cytogenetic abnormalities) do not show statistical significance.

HbF indicates hemoglobin F.

patients with all *Cbl* gene family mutations were analyzed, no distinct clinical features including differences in outcomes were found.

Discussion

The molecular pathogenesis of the often heterogeneous myeloid malignancies is not discernable through traditional morphologic analyses. Conversely, various molecular mechanisms can lead to similar clinical phenotypes and distinct mutational steps can result in various types of functional defects, each requiring distinct therapeutic approaches. Although JMML is associated with mutations in *PTPN11* and *RAS* in a large proportion of cases[3-5,12,13] and mutations of *NF-1* in a smaller fraction,[1,6,7] no specific mutations can be identified in a number of children affected by this disease.

Previously, we identified UPD11q and associated homozygous *c-Cbl* mutations in patients with CMML and secondary AML with monocytoid features.[23] We have also noted that heterozygous mutations of other closely related E3 ULs such as *Cbl-b* and *Cbl-c* may be found in some patients with otherwise indistinguishable morphologic features; these mutations presented in heterozygous constellation as they were not associated with corresponding areas of somatic UPD.[24] We have also found a significant proportion of CMML cases with UPD4q and microdeletions corresponding to the location of *TET2* gene. We have shown that UPD4q is associated with *TET2* mutations but, unlike for *c-Cbl*, heterozygous *TET2* mutations were common.[22]

Based on our progress in CMML, in this article we undertook the molecular analysis of cytogenetic abnormalities and mutational events in the clinically similar syndrome of JMML occurring in children. Using SNP-A analysis we show that patients with JMML, in addition to known typical chromosomal defects, harbor invariant somatic copy-neutral loss of heterozygosity, in particular UPD11q23.3. Based on this finding and the previously shown association of UPD11q with *c-Cbl* mutation, we demonstrated that *c-Cbl* mutations located in the RFD of this gene are found in 5 (10%) of 49 of JMML patients. Since submission of this paper, similar results were reported by Loh et al.[27] Unlike in adult CMML, *TET2* mutations were not identified in JMML, a finding consistent with the absence of UPD4q or del4 in JMML.

Our findings suggest that selective pressure in JMML leads to use of functionally related pathways but may involve distinct genes. In fact, both *c-Cbl* (ubiquitination) and *PTPN11* (dephosphorylation) mutations can lead to the augmentation of growth factor receptor–mediated signals and may explain why GM-CSF hypersensitivity is present in patients with JMML irrespective of whether *c-Cbl, PTPN11,* or *RAS* is mutated.

For *Cbl* mutations, in addition to the impaired degradation of activated growth factor receptors, altered ζ-chain–associated protein kinase 70 activation by c-Cbl may mediate proliferative signals analogous to RAS. Moreover, by binding to Grb2, c-Cbl competes with the guanine-nucleotide-exchange factor son-of-sevenless, thereby blocking signaling through the RAS–mitogen-activated protein kinase pathway and inhibiting proliferation.[25] In agreement with this theory, RFD mutant knock-in mouse experiments suggest that *c-Cbl* deprived of its E3 ligase activity may act as an oncogene, and functional analysis of mutated *c-Cbl* showed that mutated *c-Cbl* has an oncogenic effect.[35] These findings conclusively prove the pathogenic role of *c-Cbl* mutation in hematologic malignancies.

Our earlier studies showed that *c-Cbl* mutations stem from a somatic event and are not present in germline[23]; however, germline DNA was not available from our patients to conduct confirmatory studies. Nevertheless, *c-Cbl* mutations in JMML were similar or identical to those previously shown in CMML, for which the somatic nature has been confirmed through analysis of germline DNA and serial studies. Similarly, *c-Cbl* mutations were present exclusively in the context of UPD11q23.3, shown to occur only as a clonal somatic event. In agreement with a previous report,[7] we have also found UPD17q in association with neurofibromatosis-associated JMML.

Patients with *c-Cbl* mutations show comparable survival as those without *c-Cbl* mutations, but a large fraction of these patients underwent transplantation. However, *c-Cbl* mutations were associated with a younger age of presentation and smaller percentage of HbF. Given that in previous reports an older age at diagnosis and elevated HbF level have been repeatedly described as risk factors for survival in JMML,[1,36-42] lack of these poor prognostic markers in *c-Cbl* patients who demonstrate a similar outcome argues for an unfavorable impact of *c-Cbl* mutation, analogous to adult patients with *c-Cbl*.

In summary, our study describes a novel molecular lesion in children affected by JMML, suggesting similarity in the pathogenesis of a portion of patients with JMML to those with CMML.

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Authorship

Contribution: H. Muramatsu and H. Makishima designed research, performed research, analyzed data, and wrote the paper; A.M.J. and H.C. performed research; C.O. designed research, analyzed data, and wrote the paper; N.Y., Y.X., N.N., A.H., H.Y., Y.T., K.K., and A.M. designed research; S.K. designed research and wrote the paper; and J.P.M. designed research, performed research, analyzed data, and wrote the paper.

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Mutations in the ribosomal protein genes in Japanese patients with Diamond-Blackfan anemia

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ABSTRACT

Background

Diamond-Blackfan anemia is a rare, clinically heterogeneous, congenital red cell aplasia: 40% of patients have congenital abnormalities. Recent studies have shown that in western countries, the disease is associated with heterozygous mutations in the ribosomal protein (RP) genes in about 50% of patients. There have been no studies to determine the incidence of these mutations in Asian patients with Diamond-Blackfan anemia.

Design and Methods

We screened 49 Japanese patients with Diamond-Blackfan anemia (45 probands) for mutations in the six known genes associated with Diamond-Blackfan anemia: *RPS19*, *RPS24*, *RPS17*, *RPL5*, *RPL11*, and *RPL35A*. *RPS14* was also examined due to its implied involvement in 5q- syndrome.

Results

Mutations in *RPS19*, *RPL5*, *RPL11* and *RPS17* were identified in five, four, two and one of the probands, respectively. In total, 12 (27%) of the Japanese Diamond-Blackfan anemia patients had mutations in ribosomal protein genes. No mutations were detected in *RPS14*, *RPS24* or *RPL35A*. All patients with *RPS19* and *RPL5* mutations had physical abnormalities. Remarkably, cleft palate was seen in two patients with *RPL5* mutations, and thumb anomalies were seen in six patients with an *RPS19* or *RPL5* mutation. In contrast, a small-for-date phenotype was seen in five patients without an *RPL5* mutation.

Conclusions

We observed a slightly lower frequency of mutations in the ribosomal protein genes in patients with Diamond-Blackfan anemia compared to the frequency reported in western countries. Genotype-phenotype data suggest an association between anomalies and *RPS19* mutations, and a negative association between small-for-date phenotype and *RPL5* mutations.

Key words: protein genes, Diamond-Blackfan anemia, *RPL5* mutation.

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Introduction

Diamond-Blackfan anemia (DBA, MIM#105650) is a congenital, inherited bone marrow failure syndrome, characterized by normochromic macrocytic anemia, reticulocytopenia, and absence or insufficiency of erythroid precursors in normocellular bone marrow.[1] DBA was first reported by Josephs in 1936 and defined as a distinct clinical entity 2 years later by Diamond and Blackfan. Recent investigations have shown that the cellular defect in DBA fibroblasts is primarily caused by reduced proliferation and a prolonged cell cycle corresponding to the bone marrow characteristics of DBA.[2] DBA is a rare disease with a frequency of two to seven cases per million live births and has no ethnic or gender predilection.[1]

Approximately 90% of affected patients typically present in infancy or early childhood, although patients with a 'non-classical', mild phenotype are diagnosed later in life.[3,4] Macrocytic anemia is a prominent feature of DBA, but the disease is also characterized by growth retardation and congenital anomalies, including craniofacial, upper limb/hand, cardiac, and genitourinary malformations that are present in approximately half of the patients.[3-5] In addition, DBA patients have a predisposition to malignancies including acute myeloid leukemia, myelodysplastic syndrome, and osteogenic sarcoma.[3] The diagnosis of DBA is often difficult because incomplete phenotypes and wide variability of clinical expression are present.[4-6] The central hematopoietic defect is enhanced sensitivity of hematopoietic progenitors to apoptosis along with evidence of stress erythropoiesis, including elevations in fetal hemoglobin and mean red cell volume.[2] The majority of patients have an increase in erythrocyte adenosine deaminase activity.[7]

Proteins are universally synthesized in ribosomes. This macromolecular ribonucleoprotein machinery consists of two subunits: one small and one large. The mammalian ribosome comprises four RNA and 80 ribosomal proteins.[8] The first genetic anomaly identified in DBA involves the *RPS19* gene, which is mutated in approximately 25% of DBA patients. This gene is located at chromosome 19q13.2 and encodes a protein belonging to the small subunit of the ribosome.[9,10] Haploinsufficiency of the *RPS19* gene product has been demonstrated in a subset of cases[11] and appears to be sufficient to cause DBA. The RPS19 protein plays an important role in 18S rRNA maturation and small ribosomal subunit synthesis in human cells.[12,13] Deficiency of RPS19 leads to increased apoptosis in hematopoietic cell lines and bone marrow cells. Suppression of *RPS19* inhibits cell proliferation and early erythroid differentiation but not late erythroid maturation in RPS19-deficient DBA cell lines.[14]

Mutations in two other genes, *RPS24* and *RPS17*, encoding proteins of the small ribosomal subunits have been found in approximately 2% of patients.[15,16] Furthermore, mutations in genes encoding large ribosomal subunit-associated proteins, *RPL5*, *RPL11* and *RPL35A*, have been reported in 9% to 21.4%, 6.5% to 7.1%, and 3.3% of patients, respectively.[17-19] To date, approximately 50% of DBA patients in western countries have been found to have a single heterozygous mutation in a gene encoding a ribosomal protein.[1,3] These findings imply that DBA is a disorder of ribosome biogenesis and/or function. However, there have been no studies of the incidences of these mutations in Asian DBA patients.

In this study, we screened 49 Japanese DBA patients (45 probands) for mutations of the six known DBA genes and *RPS14*, which has been implicated in the 5q- syndrome, a subtype of myelodysplastic syndrome characterized by a defect in erythroid differentiation.[20]

Design and Methods

Patients

Forty-nine patients were studied in order to define the frequency and type of mutations of ribosomal protein genes associated with DBA in Japan. Eight patients were from families with more than one affected member, whereas 41 were from families with only one affected patient. The diagnosis of DBA was based on the criteria of normochromic, often macrocytic anemia; reticulocytopenia; a low number or lack of erythroid precursors in bone marrow; and, in some patients, congenital malformations, without known causes of single cytopenia including acquired or congenital infection, transient erythroblastopenia of childhood, metabolic disorders, malignancies, or autoimmune diseases. All clinical samples were obtained with informed consent from 28 pediatric and/or hematology departments throughout Japan. Additional information was obtained by a standardized questionnaire including information on birth history, age of onset or diagnosis, family history, physical examination (especially regarding malformations), hematologic data, response to therapeutic procedures, and prognosis. This study was approved by the Ethics Committee of Hirosaki University Graduate School of Medicine.

Ribosomal protein gene analysis

DNA was extracted from peripheral blood using a standard proteinase K, phenol and chloroform protocol.[21] A polymerase chain reaction (PCR) was used to amplify fragments from genomic DNA using primer sets designed to amplify the coding exons and exon/intron boundaries of the *RPS19*, *RPS17*, *RPS24*, *RPS14*, *RPL5*, *RPL11* and *RPL35A*. PCR products were directly sequenced in the forward and/or reverse direction using the ABI PRISM BigDye Terminator Cycle Sequencing Ready Reaction Kit (Applied Biosystems, Tokyo, Japan) on an ABI PRISM 310 Genetic Analyzer (Applied Biosystems, Foster City, CA, USA). *RPS19* was analyzed by determining the genomic DNA sequence of the non-coding first exon, with flanking regions, and the 450-base pair (bp) sequence upstream of the first exon (5'UTR) for each DNA sample, as previously described.[5]

To clarify the sequence of heterozygous insertion/deletion sequence variations, the respective PCR products were cloned into a TA pCR 2.1 vector (Invitrogen, Carlsbad, CA, USA) and their sequences were confirmed.

Genotype-phenotype correlations and statistical analysis

Physical abnormalities in the Japanese DBA patients were evaluated from a viewpoint of correlations with genotype. Although growth retardation can be modified by several factors such as steroid therapy, chronic anemia, and iron overload, the retardation was considered pathognomonic for DBA if it was marked, being below -3 standard deviations (SD). Response to treatment is usually seen within 1 month of treatment in DBA, but a prediction of response has not been reported previously.[1,3] We, therefore, also examined the correlation between genotype and response to the first round of steroid therapy. Associations between two groups of variables were assessed with Fisher's exact test. All tests were two-sided and P levels less than 0.05 were considered statistically significant. Data were analyzed with SPSS 11.0J software (SPSS Inc., Chicago, IL, USA).

Results

Patients' characteristics

Overall, 49 patients (45 probands) were available for analysis. The male to female ratio was 1:1.2. Forty-one index cases were classified as sporadic without unexplained anemia in first-degree relatives, while the remaining eight patients were from four families. All patients were Japanese except two cases: case 10 was Chinese and case 23 was a Brazilian of Japanese extraction. Case 15 had a Filipina mother and a Japanese father.

Genetics

RPS19

Five different mutations were detected in five probands out of 45 families (11%) (Table 1). The median age at presentation of the index cases with *RPS19* mutations was 1 month (range, 0 to 2 months). There appears to be a lower percentage of *RPS19* mutations in Japanese DBA patients than in patients from western countries. All mutations were in the coding region of the gene. Missense mutations resulting in amino acid substitutions were noted in four index cases. The three mutations, p.R62Q in case 30, p.R62W in case 44 and p.0 in case 43, have been reported in seven, ten and two families, respectively,[6,10,11,22-26] whereas one mutation, p.G95V in case 25, was novel, and could not be found in the Single Polymorphism Database (dbSNP at *www.ncbi.nlm.nih.gov/SNP*). Furthermore, the mutation was not observed in DNA from 50 normal individuals. An insertion of one nucleotide was found in one case (case 28), resulting in a novel frameshift mutation.

RPL5 and RPL11

The human *RPL5* gene consists of eight exons and is located on chromosome 1. Four novel mutations were found among the 45 probands (9%) (Table 1). The median age at presentation of the index cases with *RPL5* mutations was 10 months. A deletion of two nucleotides was found in case 10, and an insertion of one nucleotide was found in case 65, each affecting the reading frame. Two cases (cases 41 and 55) had point mutations that resulted in a loss of the translation initiation codon.

The human *RPL11* gene, which consists of six exons, is also located on chromosome 1. All exons and exon/intron boundaries were PCR-amplified and sequenced in DBA patients who were negative for mutations in *RPS19* and *RPL5*. Two mutations (4%) were found, and they were diagnosed at 18 and 20 months old, respectively (Table 1). A deletion of two nucleotides was found in case 9, and a deletion of one nucleotide was found in case 23, in each patient leading to a shift in the reading frame and the introduction of a premature stop codon.

RPS17

The *RPS17* gene is located on chromosome 15, and consists of five exons. *RPS17* mutations are rare and have been reported in only two patients with DBA. A novel one-nucleotide deletion in *RPS17* was identified in one patient (2%), resulting in the introduction of a premature stop codon (Table 1). The patient with the *RPS17* mutation (case 56) was born to healthy non-consanguineous parents and diagnosed as having DBA at the age of 1 month. He responded to the initial steroid treatment, and had a course of steroid-dependent therapy. No physical anomalies were seen in this patient.

RPL35A, RPS24 and RPS14

Mutations in *RPS24* and *RPL35A* are rare and have been reported in only eight and six patients with DBA, respectively. DBA patients were screened for *RPS24* and *RPL35A*, in addition to *RPS14*, which is implicated in the 5q- syndrome. No mutations were detected in *RPS24*, *RPL35A* or *RPS14* in Japanese DBA patients.

In total, sequence changes were found in four out of seven screened ribosomal protein genes (Table 2). Mutations in *RPS19*, *RPS17*, *RPL5*, and *RPL11* were detected in 11%, 2%, 9%, and 4% of the probands, respectively. The frequency of ribosomal protein gene mutations in Japanese DBA patients was 27%.

Genotype-phenotype correlations: congenital anomalies

The patients' characteristics are summarized in Table 3.

Table 1. **Mutations identified in *RPS19*, *RPL5*, *RPL11*, and *RPS17* in Japanese DBA patients.**

Patients (gender)	Inheritance	Age at diagnosis	Mutation	Predicted amino acid change
Mutations in the *RPS19* gene				
43 proband (F)	Sporadic	0 D	Exon2:g.3G>A	p.0
28 proband (M)	Sporadic	6 D	Exon3:g.130_131insA	E44fsX50
44 proband (F)	Sporadic	1 M	Exon4:g.184C>T	R62W
30 proband (F)	Familial	1 M	Exon4:g.185G>A	R62Q
30 father (M)	Familial	0 M	Exon4:g.185G>A	R62Q
25 proband (M)	Sporadic	2 M	Exon4:g.284G>T	G95V
Mutations in the *RPL5* gene				
10 proband (F)	Sporadic	0 M	Exon5:g.473_474delAA	K158fsX183
41 proband (F)	Sporadic	1 Y	Exon1:g.3G>T	p.0
55 proband (F)	Sporadic	3 Y	Exon1:g.3G>A	p.0
65 proband (F)	Sporadic	4 M	Exon2:g.37_38insT	F13fsX14
Mutations in the *RPL11* gene				
9 proband (F)	Sporadic	1 Y 10 M	Exon2:g.58_59delCT	L20fsX53
23 proband (M)	sporadic	1 Y 6 M	Exon5:g.460delA	R154fsX189
Mutations in the *RPS17* gene				
56 proband (F)	Sporadic	1 M	Exon2:g.26delT	V9fsX17

Anomalies associated with DBA were found in 27 patients (55%). Sixteen had two or more malformations (33%). All six patients with an *RPS19* mutation had physical anomalies, and three of them had multiple anomalies. In contrast, clinical data from European and American DBA patients showed that the frequency of malformations was 31% in patients with *RPS19* mutations, which is not significantly different from that of the entire DBA population.[26] *RPS19* mutations are characterized by a wide variability of phenotypic expression.[26] A mutation is frequently associated with various degrees of anemia, different responses to treatment, and dissimilar malformations. Even various family members having the same mutation in *RPS19* present with different clinical expressions. Cases 30, 44 and 43 harbored the same *RPS19* mutations reported in multicase families (p.R62Q, p.R62W, p.0).[6,10,11,22-27] Comparable to previous observations, no consistent clinical features were found in patients from different families displaying mutations in *RPS19*. For example, the father of case 30 harboring the same mutation had no finger anomalies, although case 30 had syndactyly and thumb polydactyly.

Consistent with reports that patients with *RPL5* and *RPL11* mutations are at high risk of developing malformations,[17,18] all four patients with *RPL5* mutations had physical anomalies. Furthermore, three of them had multiple physical anomalies, particularly case 41, who had very severe congenital heart disease (Table 3). One of two patients with *RPL11* mutations had physical anomalies. In contrast, of the 36 patients with no mutations, physical anomalies were seen in 16 (44%).

Nine patients had craniofacial anomalies. Of these, two had *RPL5* mutations, while the remaining patients had no mutations. Gazda *et al.* suggested an association between *RPL5/RPL11* mutation and cleft lip and/or palate.[17] Data in the Diamond-Blackfan Anemia Registry (DBAR) of North America also suggest that the DBA phenotype associated with cleft lip/palate is caused by non-*RPS19* mutations.[4] In our cohort, the frequency of cleft palate was significantly different between *RPL5*-mutated and *RPL5* non-mutated groups ($P<0.05$): cleft palate was seen in three patients, two of whom had *RPL5* mutations while the other patient belonged to the *RPL5* non-mutated group.

Thumb anomalies were seen in six patients, four of whom had *RPS19* mutations while two had *RPL5* mutations. There was a statistically significant difference in the frequency of thumb anomalies between *RPS19*-mutated and *RPS19* non-mutated groups ($P<0.05$). Flat thenar was seen in one patient with an *RPL5* mutation. In contrast to previous reports on patients with *RPL11* mutations, thumb anomalies were not found in our patients with these mutations.

A small-for-date phenotype was seen in seven patients (14%): one had an *RPS19* mutation, one had an *RPL11* mutation, and the four others had no mutations. None of the patients with *RPL5* mutations was born small-for-date.

Genotype-phenotype correlations: therapeutic response

Corticosteroids and transfusions are the mainstays of DBA treatment.[1,3] Of 45 patients evaluable for first treatment response, 73% responded to steroid therapy, 8% did not respond and 16% were never treated with steroids. The proportions of patients who responded to the first steroid treatment were 5/5 (*RPS19*), 2/3 (*RPL5*), 1/2 (*RPL11*), 1/1 (*RPS17*), and 22/27 (no mutation). There were no significant differences in the response rates among these patients.

Sixty-nine percent of patients received red blood cell transfusions. Of 48 patients available for therapy in follow-up, 8 patients (17%) were transfusion-dependent, 18 patients (37%) were steroid-dependent, and 18 patients (37%) were transfusion-independent with no other treatment. Three patients received bone marrow transplants and were alive and well (Table 3). A malignancy was detected in one case (case 50, proband), who developed a myelodysplastic syndrome 1 year after the diagnosis of DBA.

Table 2. **Summary of sequence changes in seven ribosomal protein genes identified in Japanese DBA patients.**

Gene symbol	N. of tested DNA samples from unrelated probands	N. of probands with mutations	N. of subjects with mutations	Mutation types
RPS19	45	5 (11%)	6	missense, loss of 1st methionine, small insertion
RPL5	45	4 (9%)	4	loss of 1st methionine, small deletion, small insertion
RPL11	34	2 (4%)	2	small deletion
RPS17	45	1 (2%)	1	small deletion

Discussion

This is the first report of an investigation of DBA patients in Japan. Twelve types of mutations were detected in four ribosomal protein genes. These mutations occurred in 27% of Japanese DBA patients. Mutations in *RPS19*, which have been found in 25% of patients in western countries,[26] were detected in only five of 45 probands (11%) in Japan, and two of these mutations were unique. Novel mutations in *RPL5* (four probands; 9%), *RPL11* (two probands; 4%) and *RPS17* (one proband; 2%) were identified. The frequencies of mutations in *RPL5*, *RPL11* and *RPS17* were very similar to those in western countries.[16-19] These results may suggest that a lower incidence of mutations in ribosomal protein genes in Japanese patients with DBA is due to a lower incidence of *RPS19* mutations, although we might have missed large deletions or re-arrangements in this study.

Physical abnormalities and growth retardation were detected in 55% of the Japanese DBA patients, consistent with previous reports from western countries.[4-6] Recent studies suggest that patients with *RPL5* mutation are more likely to have physical malformations including craniofacial, thumb, and heart anomalies.[17,18] Remarkably, patients with *RPL5* mutations tend to have cleft lip and/or palate or cleft soft palate, isolated or in combination with other physical abnormalities.[17,18] We found that three of four patients with *RPL5* mutations had multiple physical malformations, and two had cleft palate, whereas only one patient without an *RPL5* mutation had cleft palate. In the general population, 0.1% to 0.2% of children are born with cleft lip and/or palate.[28] Our data, and those from previous findings, suggest that *PRL5* mutations are associ-

Table 3. **Characteristics of Japanese DBA patients.**

Patient	Malformation status	Response to first steroid therapy	Present therapy
Patients with mutation of *RPS19*			
25 proband	Thumb polydactyly, growth retardation (-2.0SD), etc.	ND	ND
28 proband	Thumb polydactyly, CHD, etc.	Response	Steroid-dependent
30 proband	Thumb polydactyly, syndactyly, growth retardation (-3.4SD)	Response	Steroid-dependent
30 father	Growth retardation (-3.6SD)	NA	CR
43 proband	Thumb polydactyly	Response	Steroid-dependent
44 proband	SFD	Response	CR
Patients with mutation of *RPL5*			
10 proband	Flat thenar, cleft palate, CHD, etc.	Poor	Transfusion-dependent
41 proband	Craniofacial abnormalities, cleft palate, CHD, etc.	ND	Transfusion-dependent
55 proband	Thumb polydactyly	Response	Steroid-dependent
65 proband	Growth retardation (-3.0SD)	Response	Steroid
Patients with mutation of *RPL11*			
9 proband	CHD, SFD, etc.	Response	CR
23 proband	None	Poor	Steroid-dependent
Patient with mutation of *RPS17*			
56 proband	None	Response	Steroid-dependent
Patients without mutation of seven RP genes			
1 proband	Growth retardation (-4.0SD)	Response	CR
1 daughter	None	Response	CR
3 proband	Growth retardation (-3.6SD)	Response	Steroid-dependent
4 proband	Craniofacial abnormalities, SFD, short stature, webbed neck	Response	Steroid-dependent
5 proband	None	Response	CR
6 proband	Cleft palate, SFD, etc.	Poor	BMT
7 proband	Craniofacial abnormalities, SFD, growth retardation, etc.	Response	CR
8 proband	Growth retardation, webbed neck	Response	Steroid-depndent
13 proband	None	NA	CyA, BMT
14 proband	None	Response	CR
15 proband	None	Response	Transfusion-dependent
20 proband	Craniofacial abnormalities, CHD, etc.	Rsponse	Transfusion-dependent
21 proband	None	Response	Steroid-dependent
22 proband	None	Response	CR
24 proband	Growth retardation (-4.0SD)	Response	Steroid-dependent
26 proband	Growth retardation (-4.1SD), craniofacial abnormalities, etc.	Response	Transfusion-dependent
33 proband	None	Response	BMT
36 proband	Hypospadias, cryptorchidism	Response	Steroid-dependent
36 cousin	None	Response	Steroid-dependent
37 proband	Hypospadias, cryptorchidism	ND	CR
42 proband	None	Response	CR
45 proband	Craniofacial abnormalities, growth retardation, etc.	Poor	Transfusion-dependent
48 proband	Fetal hydrops	ND	CR
49 proband	None	Response	Steroid-dependent
50 proband	None	Response	Steroid-dependent, CBT (due to MDS)
50 sister	None	Response	Steroid-dependent
51proband	None	Poor	CR
54 proband	None	ND	Transfusion-dependent
59 proband	None	ND	Transfusion
60 proband	SFD	ND	Transfusion
61 proband	None	Response	Cyclosporine
62 proband	CHD, SFD, growth retardation (-3.1SD)	Response	Steroid-dependent
63 proband	Craniofacial abnormalities, growth retardation (-7.5SD)	Response	Steroid-dependet
64 proband	None	Response	Steroid-depndent
66 proband	None	NA	Transfusion-dependent
67 proband	None	NA	NA

*ND: not done; NA: not available; SFD: small-for-date; CHD: congenital heart disease; MDS: myelodysplastic syndrome; BMT: bone marrow transplantation; CBT: cord blood stem cell transplantation; CR: complete remission. * RPS19, RPS24, RPS17, RPS14, RPL5, RPL11, RPL35A.*