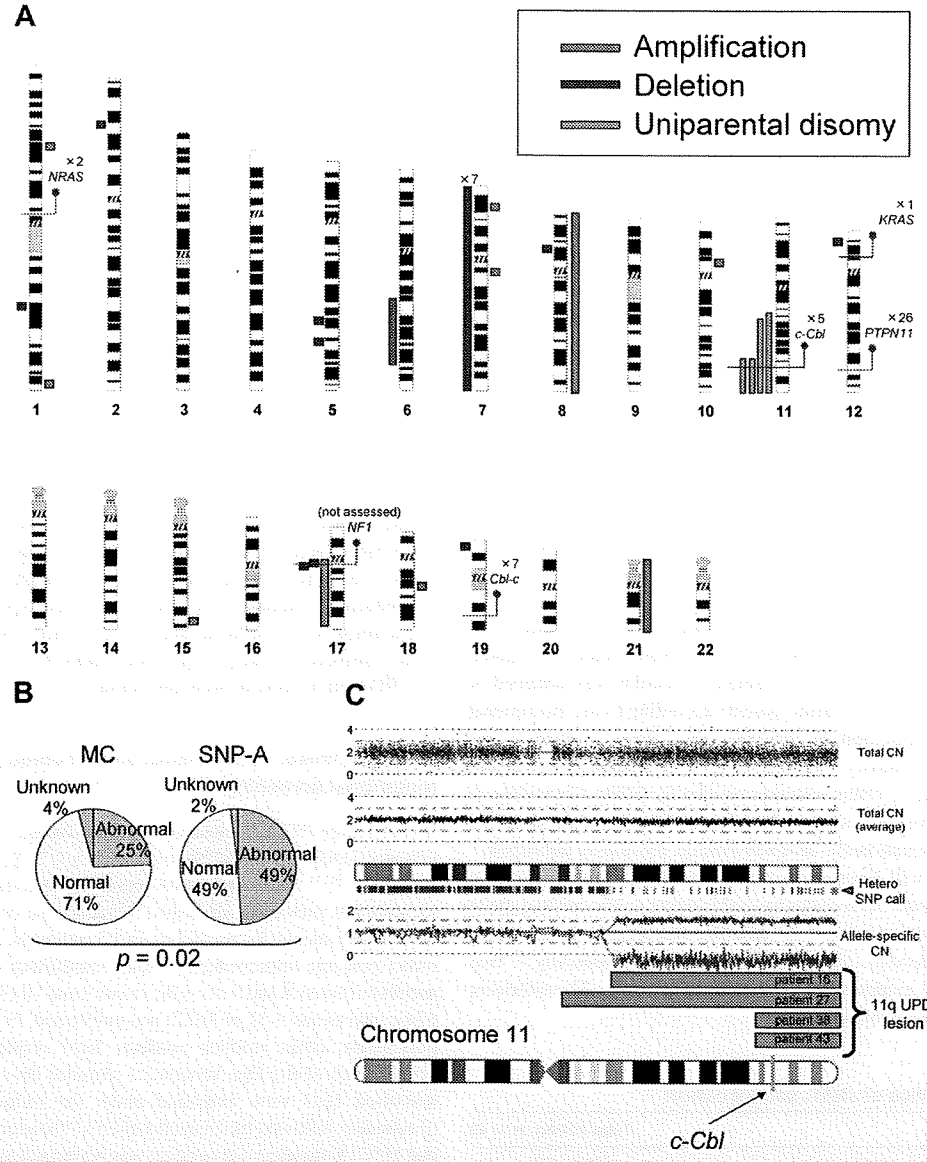


Figure 1. Single nucleotide polymorphism array–based karyotyping of JMML. (A) Genomic distribution and type of lesion identified in patients with JMML by SNP-A analysis. Green bar represent amplification, red shows deletion, and blue corresponds to UPD. Red lines pinpoint the locus of genes discussed 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.

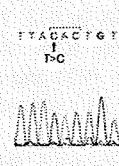
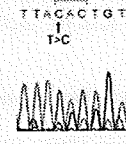
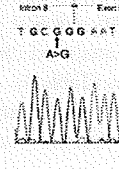
Mutational analysis of patients with JMML

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

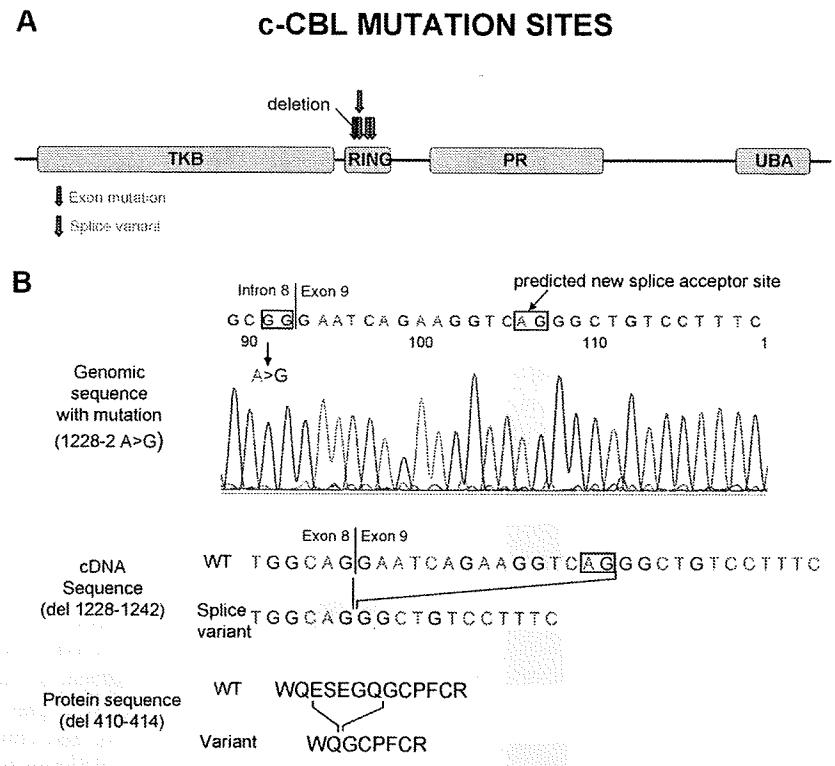


Figure 2. Site of the *c-Cbl* mutations and predicted product of splice variant in the intron 8 splice acceptor site. (A) Localization of the c-Cbl mutations within the predicted protein product. Red arrows show the site of mutations in exon, and blue arrows show the site of splice variant. (B) In patient 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

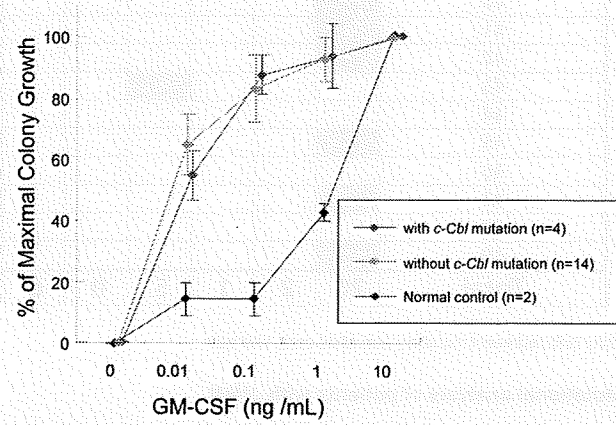


Figure 3. GM-CSF hypersensitivity assay. Colony counts are expressed as percentage of maximal numbers of CFU-GM (colony counts cultured with each concentration of GM-CSF/colony counts cultured with 10 ng/mL GM-CSF). The similar GM-CSF hypersensitivity was seen in JMML patients with or without *c-Cbl* 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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Relapse of leukemia with loss of mismatched HLA due to uniparental disomy following haploidentical hematopoietic stem cell transplantation

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Running title: Leukemic relapse with HLA loss after haplo SCT

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Abstract

We investigated 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 three 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 six 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.

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 were related to the relapse after HLA-identical or haploidentical HSCT.

Materials and Methods

Patients and transplantation procedure

We identified nine 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 Table S1. Three patients received HSCT from an HLA-haploidentical family donor and the other six patients received HSCT from an HLA-matched donor (four siblings and two 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-transplant 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 from Medical&Biological Laboratories (Nagoya, Japan); HLA-A11 (IgM), HLA-A30, -31 (IgM), HLA-25, -26 (IgM), HLA-Bw6 (IgG3),

and HLA-Bw4 (IgG3) antibodies from One Lambda (Canoga Park, CA) were purchased. For leukemic cell markers, CD13-PE (IgG1) from Immunotech (Marseille, France) and CD34-APC (IgG1) from BD (San Jose, CA) were purchased. Samples were analyzed with FACSCalibur cytometer and CellQuest software (BD, San Jose, CA). 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, Tokyo, Japan) 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 cytotoxic T-lymphocyte precursor 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]

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 (1×10^4 cells/well) were mixed with the indicated stimulator cells (1×10^4 cells/well) in 96-well, round-bottom polypropylene plates and spun at 1,200 rpm for 3 minutes before overnight incubation in 200μl of RPMI-1640 medium supplemented with 10% fetal bovine serum. On the next day, 50μl of supernatant was collected and interferon (IFN)-γ was measured by enzyme-linked immunosorbent assay with 3,3',5,5'-tetramethylbenzidine substrate (Sigma-Aldrich, St. Louis, MO).

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 three courses of donor lymphocyte infusion (DLI) for relapsed leukemia after haploidentical HSCT. After the third unmanipulated-DLI (1×10^7 $CD3^+$/kg), she experienced acute grade-III graft-versus-host disease (GVHD) 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 two of three patients who underwent HLA-haploidentical HSCT, while microscopic analysis showed relapse (Fig. 1A). To test whether HLA class-I molecules could be upregulated, samples were cultured for 48-h in medium supplemented with TNF-α or IFN-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 not only the HLA-A locus but also the HLA-B, -C, and –DR loci were not encoded; only the mismatched haplotype was lost in both patients (Table S2). 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 (Fig. S1). These results suggest that loss of HLA class-I haplotype at the time of post-transplant 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 (Fig. 1B). Loss of one allele from one parent and duplication of the remaining allele from the other parent leads to UPD.[14]

In Patient 2, we examined if the number of CTLp had changed during the post-transplant 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 following three 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 721.221 B-lymphoblastoid cell line transfected with one of three mismatched HLA-alleles (Fig. 1C, D).

Despite high transplant-related mortality due to severe GVHD and post-transplant 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, two of three 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, . 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] Immunological 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 two 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 (Table S2). 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 downregulation of HLA on relapsed leukemic blasts after HLA-haploidentical HSCT should be considered, because DLI would likely be ineffective in patients whose leukemic cells lose HLA class-I antigen.

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Authorship contributions

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. All other authors were responsible for clinical work and critically reviewed the manuscript.

Conflict of interest disclosure

All authors have no conflict of interest to declare.

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Table 1. The cytotoxic T-lymphocyte precursor frequency reactive to the recipient alloantigen in the recipient after transplantation and the donor

Samples	Max $CD8^+$ input*	Number of growing wells†	CTLp frequency^{-1} (95% confidence interval)
Donor	33,300	8	8.6×10^5 ($1.49 \times 10^6 - 5.0 \times 10^5$)
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 ($7.2 \times 10^5 - 2.5 \times 10^5$)

* 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 donor lymphocyte infusion or 2

weeks after complete remission was confirmed by bone marrow aspirate.

UD: undetermined due to no growing wells present.

Figure Legends

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

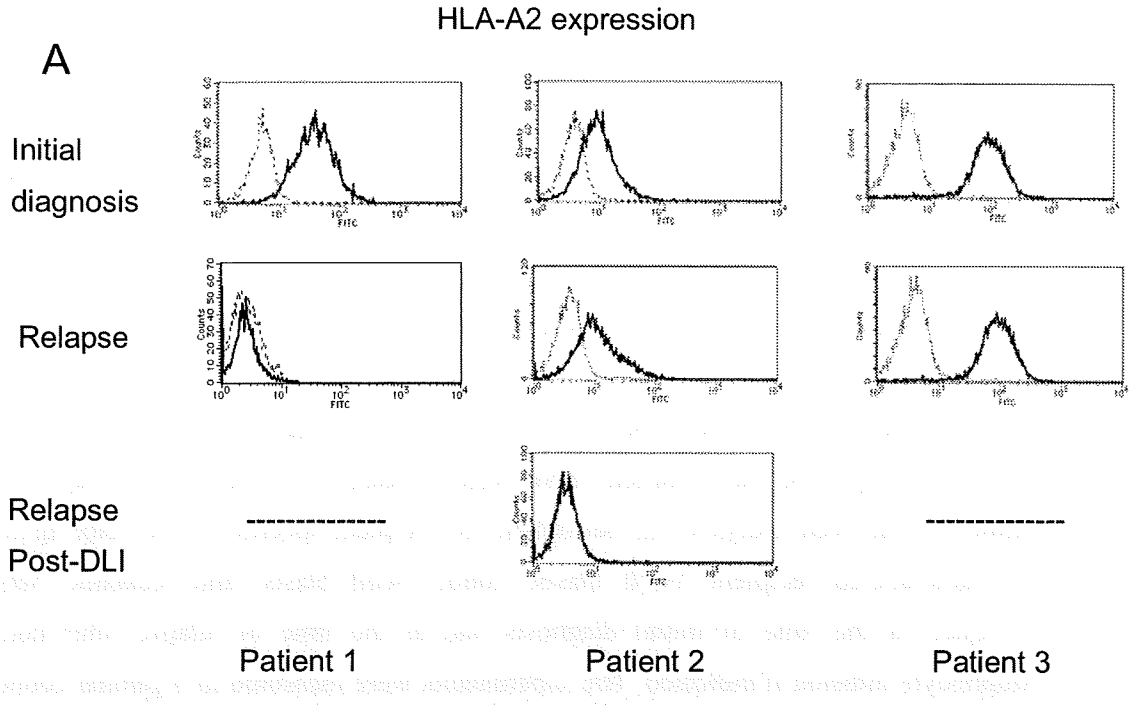
(A). Leukemic blasts at the time of initial diagnosis and at the time of relapse after hematopoietic stem cell transplantation (HSCT) and donor lymphocyte infusion (DLI) were gated by $CD34^+$ and $CD13^+$ and then the surface expression of mismatched HLA alleles was examined with anti-HLA-A2 antibodies. In three patients with acute myelogenous leukemia 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 donor lymphocyte infusion. **(B).**Single-nucleotide polymorphism 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 single-nucleotide polymorphisms (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 five 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.

Table 1. The cytotoxic T-lymphocyte precursor frequency reactive to the recipient alloantigen in the recipient after transplantation and the donor

Purified $CD8^+$ T cells from the peripheral blood mononuclear cells obtained post-transplant 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, IL-6, and IL-7 (10 ng/ml, both from R&D Systems, Minneapolis, MN). 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 donor lymphocyte infusion if indicated. The supernatants were measured in a gamma counter after 4-h incubation. The wells were considered to be positive for cytolytic activity if the total counts per minute released by effector cells was $>3 \times$ SD above the control wells (mean counts per minute released by the target cells incubated with irradiated stimulator cells alone). The cytotoxic T-lymphocyte precursor (CTLp) frequency was calculated using L-Calc software (StemCell Technologies, Vancouver, Canada). 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, while the CTLp frequency obtained at Day 520 (1 month after the third donor lymphocyte infusion 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.

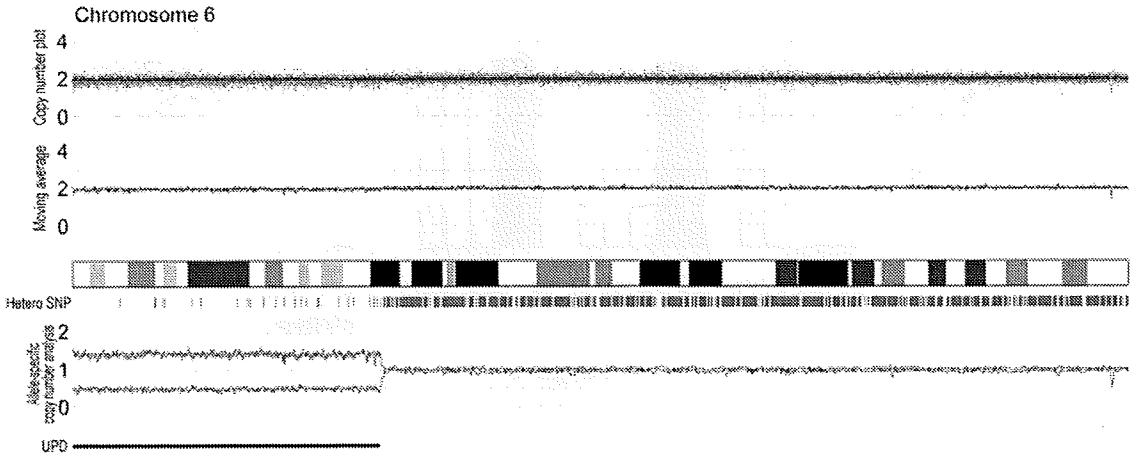
Figure 1


HLA-A2 expression
A
Initial diagnosis
Relapse
Relapse Post-DLI

Patient 1
Patient 2
Patient 3

B

Patient 1



Patient 2