Introduction

The prognosis of pediatric acute myeloid leukemia (AML) has improved markedly over the past decade, with an overall survival rate of about 60–70% according to the results of various clinical trials; however, relapse remains the major cause of treatment failure, occurring in 30–40% of patients in their first complete remission (CR) [1–6]. Several study groups have shown that chromosome abnormalities are independent and strong predictors of the outcome, both in childhood and adult AML [1, 4, 7].

It is hypothesized that AML results from two classes of cooperating mutations [8–10]. Type II mutations affect hematopoietic stem cell differentiation, typically by mutations or translocations (e.g., *AML1-ETO*, *PML-RARα*, or *CBFβ-MYH11*), resulting in the aberrant function of transcription factor complexes; however, these mutations alone are insufficient to induce leukemia. Type I mutations provide a proliferative and survival advantage to hematopoietic progenitors [11]. This group includes activating mutations in receptor tyrosine kinases (RTKs) such as *FLT3* and GTPase *RAS* [11–14].

The *RAS* family of genes consists of three G-proteins, *NRAS*, *KRAS*, and *HRAS* [15]. The RAS proteins are important in relaying proliferation and survival signals from cell membrane receptors (including KIT and FLT3) to intracellular signal transduction pathways. Certain mutations in *RAS* induce the permanent activation of RAS. Mutations in *NRAS* or *KRAS* have been identified in numerous malignancies [15], including hematologic malignancies such as AML [15–17].

Although mutations in *RAS* are frequent in AML and considered to contribute to leukemogenesis in a subset of patients, their prognostic significance has not been firmly established. Some reports have suggested that patients with AML showing *RAS* mutation have poorer [18–20] or similar clinical outcomes to patients carrying the wild-type *RAS* gene [16, 21–26], whereas others have found that mutations in *RAS* are associated with a more favorable prognosis [17, 27]. Although these conflicting results may stem from variations in the pretreatment features of patient populations analyzed in different series, they may also be related to differences in the treatment regimens used.

We performed mutational analysis of *RAS* in pediatric AML patients who were treated on the Japanese Childhood Cooperative Study Group Protocol, AML99, and demonstrated that *RAS* mutations were frequently found in French-American-British (FAB) types M4 and M5, and related to late relapse. Furthermore, we analyzed the association between *RAS* mutations and other gene aberrations, including *KIT* mutation, *FLT3*-ITD, and *MLL*-PTD.

Materials and methods

Patients

The diagnosis of AML was based on the FAB classification, and cytogenetic analysis was performed using a routine G-banding method. From January 2000 to December 2002, 318 patients were newly diagnosed as having de novo AML. Of these, samples from 157 patients were available for molecular analysis, including 13 with FAB-M3 and 10 with Down syndrome (DS), who were treated on different treatment protocols [5, 28–30]. There were no significant differences between the 134 patients without FAB-M3 or DS and the 106 non-analyzed patients in terms of the age [median 6 years (range 0–15 years) vs. 6 years (range 0–15 years)] and initial WBC count [median 24.8 $\times$ 10^9/L (range 1.65–621.0 $\times$ 10^9/L) vs. 13.8 $\times$ 10^9/L (range 1.0–489.0 $\times$ 10^9/L, $P = 0.0764$)]. Patients who were younger than 2 years old or had an initial WBC count <100,000/μL were treated using the induction A regimen [etoposide (VP-16), cytarabine (CA), and mitoxantrone (MIT), (ECM)]. Patients who were older than 2 years and had an initial WBC count >100,000/μL were treated using the induction B regimen [VP-16, CA, and idarubicin (IDA), (ECI)]. If patients achieved a complete remission (CR), they were classified into three risk groups (62 low, 57 intermediate, and 10 high) according to the results of cytogenetic analyses or the achievement of CR after the 2 initial courses of chemotherapy [5, 28–30]. AML patients with t(8;21) (except for those with WBC counts >50,000/μL) or inv(16)(p11q22) were classified into the low-risk (LR) group. Patients with monosomy 7, 5q-, t(16;21), or Philadelphia chromosome (Ph1) were classified into the high-risk (HR) group. Patients were treated with additional chemotherapy or allogeneic stem cell transplantation (allo-SCT) in each risk group.

Informed consent was obtained from the patients or their parents, according to guidelines based on the tenets of the revised Helsinki protocol. The institutional review board of Gunma Children's Medical Center approved this project.

Detection of *RAS* mutations

Total RNA (4 μg) extracted from the bone marrow or peripheral blood samples at diagnosis was reverse transcribed to cDNA with a cDNA Synthesis Kit (Amersham Bioscience, Tokyo, Japan). Mutations of codons 12, 13, and 61 of *NRAS* and *KRAS* genes were directly sequenced using the following primers: NRAS EX1S 5′-TACAAACT GGTGGTGGTTGG-3′; NRAS EX2R 5′-CAAATGACTT GCTATTATTGATG-3′; KRAS EX1S 5′-GGCCTGCTGA AAATGACTGAATAT-3′; KRAS EX2R 5′-ACTGGTCC CTCATTGCACT-3′.

Detection of *FLT3*-ITD, *FLT3*-D835, *KIT* mutations, and *MLL*-PTD

Mutational analysis of internal tandem duplication (ITD) within the JM domain and D835 mutation (D835Mt) within the TK2 domain of *FLT3* was performed as previously reported [28, 30–32]. Mutation analysis of the kinase domain, extracellular domain, and transmembrane domain of the *KIT* gene was performed with RT-PCR followed by direct sequencing as previously reported [28]. Mutational analysis of partial tandem duplication (PTD) of *MLL* was performed using the primer pair 6.1 (located in exon 9) and E3AS (located in exon 4) as previously reported [30].

Statistical analysis

The χ^2 test was used to compare the frequencies of mutations. Fischer's exact test was used when data were sparse. The survival distribution was assessed using the Kaplan–Meier method, and differences were compared using the log-rank test [33]. Overall survival (OS) was defined as the time from diagnosis until death owing to any cause or the last follow-up. Disease-free survival (DFS) was defined as the time from the date of complete remission until relapse. To compare survival in the relatively late course of treatment, we further measured OS and DFS from the predefined 'landmark' time, but not from the onset, in cases in first complete remission at the landmark time [34]. All patients enrolled in AML99 finished the medical treatment including hematopoietic stem cell transplantations within 1.4 years after complete remission; therefore, the landmark time was defined as 1.4 years after complete remission. In landmark analysis, both OS and DFS were calculated using the survival duration after complete remission. These statistical analyses were based on Dr SPSS II for Windows (release 11.0.1J, SPSS; Japan, Inc.).

Results

RAS mutations

Of 157 pediatric AML patients, 29 (18.5%) had an activating mutation: 10 (6.4%) in *NRAS* exon 1, one (0.6%) in *NRAS* exon 2, 13 (8.3%) in *KRAS* exon 1, and 5 (3.2%) in *KRAS* exon 2. There was no significant difference in age, sex, WBC count at diagnosis, or the frequency of extramedullary infiltration of leukemic cells between patients with and without *RAS* mutations (Table 1).

RAS mutations in cytogenetic subgroups

Fifty-three patients in this study were diagnosed with CBF-AML, including 46 patients with t(8;21) and 7 with inv(16). In this group of patients, 12 (22.6%) had a mutation in *NRAS* or *KRAS*. The incidence of *RAS* mutation in the t(8;21) and inv(16) subgroups was 21.7 and 28.6%, respectively. Thirty-three patients in this cohort had a normal karyotype, with 18.2% of these patients having a mutation in *RAS*. The incidence of mutations in *RAS* was not a significantly frequent event in pediatric CBF-AML among other cytogenetic subgroups (Table 1, in which DS patients were not included in karyotypic abnormalities).

RAS mutations in FAB subgroups

The five most prevalent FAB types in the patient cohort were M2, M1, M5, M4, and M7 in order of descending prevalence. When examining these five subgroups, the frequency of RAS mutation was 31.8% (M4), 28.0% (M5), 19.6% (M2), 12.5% (M1), and 5.3% (M7). The frequency of *RAS* mutation was higher in FAB types M4 and M5 than in the other types (29.8 vs. 13.6%, respectively, $P = 0.02$) (Table 1).

Correlations between RAS mutations and other gene aberrations

In *RAS*-mutated cases, 10 cases (34.5%) had mutations in other genes, including 1 (3.4%) in *FLT3*-ITD, 2 (6.9%) in *FLT3*-D835 mutation, 4 (13.8%) in *KIT* mutation, and 3 in *MLL*-PTD (10.3%). This distribution was not different from those without *RAS* mutation (Table 1).

Clinical outcome and prognostic significance of *RAS* mutations

There were no significant differences in the 3-year OS, DFS, and the induction rate of complete remission (96 vs. 93%, respectively, $P = 0.88$) between those with and without *RAS* mutation in 134 AML patients, excluding those with FAB-M3 and DS (Fig. 1). Between *NRAS*-mutated ($n = 11$) and *KRAS*-mutated patients ($n = 17$) excluding those with FAB-M3 and DS, there were no differences in the 3-year OS (73 vs. 77%, respectively, $P = 0.91$), DFS (73 vs. 50%, respectively, $P = 0.48$), and the induction rate of complete remission (100 vs. 94%, respectively, $P = 0.82$). The frequency of *RAS* mutation did not differ between patients with and without complete remission after induction therapy (22.0 vs. 9.1%, respectively, $P = 0.73$).

On the other hand, by the analysis of OS and DFS from the predefined 'landmark' time of 1.4 years after complete remission, patients with RAS mutation had a poorer 3-year OS ($P = 0.038$) and DFS ($P = 0.026$) than those without RAS mutation in 106 patients (OS) and 84 patients (DFS), excluding those with FAB-M3 and DS (Fig. 2a, b).

Table 1 Clinical characteristics of patients with and without RAS mutations

	All patients	RAS-Wt	RAS-Mt	NRAS-Mt	KRAS-Mt
Age, median (years)	6 (0–15)	6 (0–15)	7 (0–15)	10 (0–15)	6 (0–14)
WBC count, median ($\times 10^9$/L)	20.7 (1.0–620.0)	17.2 (1.0–620.0)	41.6 (1.8–224.4)	47.1 (2.0–198.0)	36.6 (1.8–224.4)
Male/female	89/68	71/57	18/11	9/2	9/9
Patients with Down syndrome	10 (6.4%)	9 (7.0%)	1 (3.4%)	0	1 (5.6%)
FAB classification					
M0	5 (3.2%)	4 (3.1%)	1 (3.4%)	1 (9.1%)	0
M1	24 (15.3%)	21 (16.4%)	3 (10.3%)	1 (9.1%)	2 (11.1%)
M2	46 (29.3%)[a]	37 (28.9%)[a]	9 (31.0%)	4 (36.4%)	5 (27.8%)
M3	13 (8.3%)	13 (10.2%)	0	0	0
M4	22 (14.0%)	15 (11.7%)	7 (24.1%)	3 (27.3%)	4 (22.2%)
M5	25 (15.9%)	18 (14.1%)	7 (24.1%)	2 (18.2%)	5 (27.8%)
M6	1 (0.6%)	0	1 (3.4%)	0	1 (5.6%)
M7	19 (12.1%)[a]	18 (14.1%)[a]	1 (3.4%)[a]	0	1 (5.6%)[a]
Unclassified	2 (1.3%)	2 (1.6%)	0	0	0
Other genomic alterations[b]					
FLT3-ITD	17 (10.8%)	16 (12.5%)	1 (3.4%)	1 (9.1%)	0
FLT3 D835 mutation	8 (5.1%)	6 (4.7%)	2 (6.9%)	0	2 (11.1%)
KIT mutation	12 (7.6%)	8 (6.3%)	4 (13.8%)	1 (9.1%)	3 (16.7%)
MLL-PTD	21 (13.4%)	18 (14.1%)	3 (10.3%)	1 (9.1%)	2 (11.1%)
Total	157	128	29	11	18
Karyotypic abnormalities (excluding Down syndrome)					
Normal	33 (22.4%)	27 (22.7%)	6 (21.4%)	2 (18.2%)	4 (23.5%)
t(8;21)	46 (31.3%)	36 (30.3%)	10 (35.7%)	4 (36.4%)	6 (35.3%)
11q23 abnormality	19 (12.9%)	14 (11.8%)	5 (17.9%)	1 (9.1%)	4 (23.5%)
t(15;17)	13 (8.8%)	13 (10.9%)	0	0	0
inv(16)	7 (4.8%)	5 (4.2%)	2 (7.1%)	2 (18.2%)	0
Others	27 (18.4%)	22 (18.5%)	5 (17.9%)	2 (18.2%)	3 (17.6%)
Unknown	2 (1.4%)	2 (1.7%)	0	0	0
Total	147	119	28	11	17
Risk group (excluding FAB-M3 and Down syndrome)					
Low	62 (46.3%)	49 (46.2%)	13 (46.4%)	4 (36.4%)	9 (52.9%)
Intermediate	57 (42.5%)	44 (41.5%)	13 (46.4%)	6 (54.5%)	7 (41.2%)
High	10 (7.5%)	9 (8.5%)	1 (3.6%)	1 (9.1%)	0
Non-CR	5 (3.7%)	4 (3.8%)	1 (3.6%)	0	1 (5.9%)
Total	134	106	28	11	17

[a] Of 10 cases with Down syndrome, 9 were classified into FAB-M7 and 1 was into FAB-M2. *RAS* (*KRAS*) mutation was observed in one case (FAB-M7) with Down syndrome

[b] One case with Down syndrome harboring *RAS* mutation had no other genomic alteration

Discussion

We analyzed the clinical significance of *RAS* mutation in a cohort of 157 pediatric AML patients. Some studies have reported that *RAS* mutations confer a poor prognosis [18–20]. In this study, the 3-year OS and DFS were not different between patients with and without *RAS* mutation. This discrepancy may be due to the fact that the treatment regimen used in each study was different. In adult cases, AML patients carrying a *RAS* mutation have been reported to benefit from higher cytarabine doses more than those without *RAS* mutation [35]. The current patients were treated on the Japanese Childhood AML Cooperative Study Group Protocol, AML99, in which the intensive use of cytarabine, including high-dose cytarabine, was considered to improve the outcome. Improvement of the clinical outcome of patients with *RAS* mutation in this study might have decreased the differences in the 3-year OS and DFS between patients with and without *RAS* mutation.

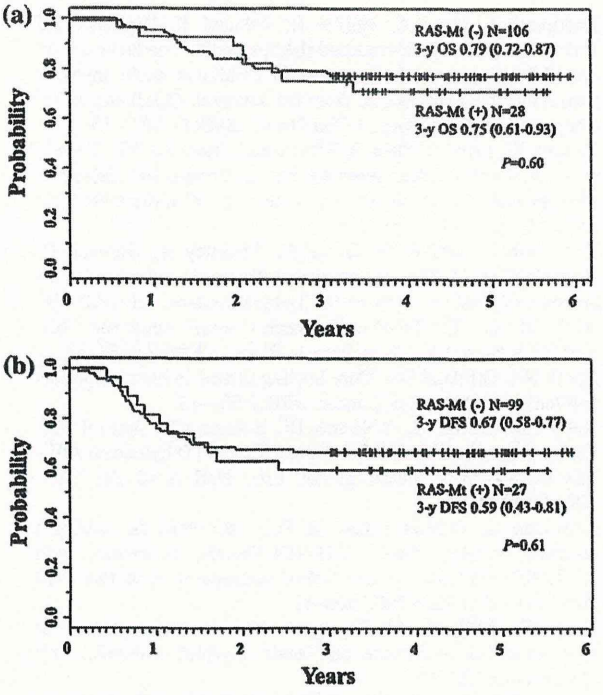

Fig. 1 Probability of 3-year OS (a) and DFS (b) in 134 AML patients, excluding those with FAB-M3 and Down syndrome. Kaplan–Meier estimates for patients with and without *RAS* mutation are shown

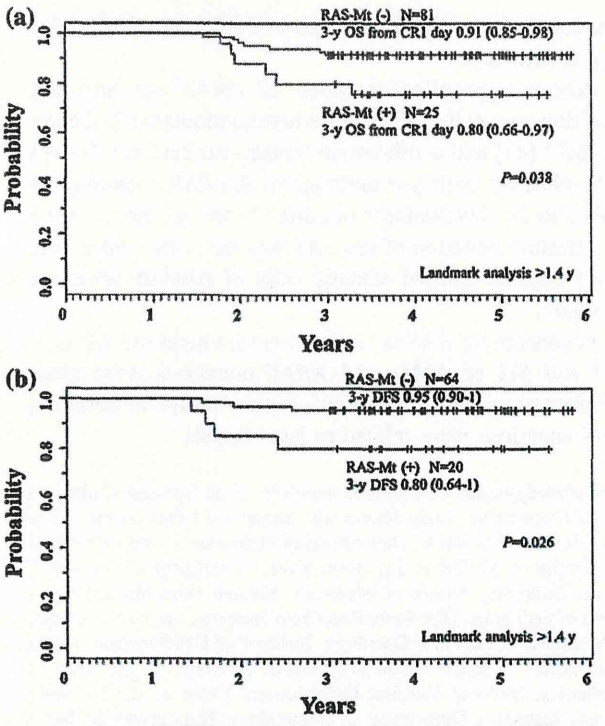

Fig. 2 Probability of 3-year OS in 106 patients (a) and DFS in 84 patients (b) from the landmark time of 1.4 years from the date of complete remission (CR1 day), excluding those with FAB-M3 and Down syndrome. Kaplan–Meier estimates for patients with and without *RAS* mutation are shown

To compare survival in the relatively late course of treatment, OS and DFS were also calculated from the predefined 'landmark' time of 1.4 years after complete remission, excluding patients with FAB-M3 and DS. As a result of landmark analysis, the 3-year OS and DFS of patients with *RAS* mutation were inferior to those without *RAS* mutation, which was different from the results of conventional analysis. This discrepancy might be due to the influence of cases with *FLT3*-ITD, since all relapses in cases with *FLT3*-ITD had taken place within 9 months [30]. This landmark analysis was meaningful in clarifying the influence of *RAS* mutation on late relapse by excluding the influence of *FLT3*-ITD on early relapse. To the best of our knowledge, no reports have discussed the meaning of *RAS* mutation in such relatively late course of treatment. Although further studies should focus on the prognostic impact of *RAS* mutation, the occurrence of death or relapse at a relatively late period should be paid attention to in AML patients with *RAS* mutation.

The incidence of *RAS* mutation in this study was 18.5%, almost the same as in previous reports [36–40]. In adult cases, *NRAS* mutations were reported to be more frequent than *KRAS* mutations [19, 41]; however, there have been few reports on pediatric AML. In pediatric cases, *KRAS* mutations were reported to be less frequent than *NRAS* mutations (18–40% in pediatric patients with *RAS* mutations) [20, 25, 42]; however, *KRAS* mutations were more frequent than *NRAS* mutations in this study (18/29, 62.1% in patients with *RAS* mutation). Although the reason for this discrepancy is not clear, this might be due to racial differences. As another possibility, the frequency of *NRAS* or *KRAS* mutation may differ between adult and pediatric patients.

The frequency of *RAS* mutation was higher in FAB types M4 and M5 than other types in this study ($P = 0.02$). Mutant *RAS* was suggested to promote a myeloid maturation defect, with relative sparing of the monocyte-macrophage lineage in vitro [43]. This may be consistent with the high incidence of *RAS* mutation in FAB types M4 and M5. In adult cases, *KRAS* mutation was reported to frequently demonstrate significant heterogeneity among FAB subtypes, being more common in M4 [24]; however, the frequency of FAB type M4 was not significantly high in patients with *KRAS* mutation in our study (22.2% of *RAS*-mutated vs. 13.3% of wild-type *RAS* samples, $P = 0.38$). In pediatric cases, the frequencies of FAB types M2 and M4 were reported to be high in patients with *RAS* mutation [25]; however, no significant differences were observed in this study (55.2% of *RAS*-mutated vs. 40.6% of wild-type *RAS* samples, $P = 0.15$). Further study is needed to clarify

the distribution of *RAS* mutation in FAB types, especially in pediatric cases.

Recently, germline mutation of *NRAS* has attracted attention as a cause of juvenile myelomonocytic leukemia (JMML) [44] and autoimmune lymphoproliferative disease [45]. Notably, germline mutation of the *RAS* gene may be linked to the development of AML; however, the presence of germline mutation of the *RAS* was not identified in this study because normal somatic cells of patients were not available.

In conclusion, *RAS* mutations were frequent in FAB type M4 and M5 of AML, and *KRAS* mutations were more frequent than *NRAS* mutations in this study. In addition, *RAS* mutations were related to late relapse.

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Conflict of interest There is no conflict of interest.

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Escape of leukemia blasts from HLA-specific CTL pressure in a recipient of HLA one locus-mismatched bone marrow transplantation

Tomonori Kato [1], Seitaro Terakura [1], Makoto Murata *, Kyoko Sugimoto, Miho Murase, Chisako Iriyama, Akihiro Tomita, Akihiro Abe, Momoko Suzuki, Tetsuya Nishida, Tomoki Naoe

Department of Hematology and Oncology, Nagoya University Graduate School of Medicine, 65 Tsurumai-cho, Showa-ku, Nagoya, Aichi 466-8550, Japan

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ABSTRACT

A case of leukemia escape from an HLA-specific cytotoxic T lymphocyte (CTL) response in a recipient of bone marrow transplantation is presented. Only the expression of HLA-B51, which was a mismatched HLA locus in the graft-versus-host direction, was down-regulated in post-transplant leukemia blasts compared with that in pre-transplant blasts. All CTL clones, that were isolated from the recipient's blood when acute graft-versus-host disease developed, recognized the mismatched B*51:01 molecule in a peptide-dependent manner. The pre-transplant leukemia blasts were lysed by CTL clones, whereas the post-transplant leukemia blasts were not lysed by any CTL clones. The IFN-γ ELISPOT assay revealed that B*51:01-reactive T lymphocytes accounted for the majority of the total alloreactive T lymphocytes in the blood just before leukemia relapse. These data suggest that immune escape of leukemia blasts from CTL pressure toward a certain HLA molecule can lead to clinical relapse after bone marrow transplantation.

1. Introduction

Allogeneic hematopoietic stem cell transplantation (HSCT) is curative for leukemia by virtue of the immune reaction mediated by donor T lymphocytes, termed the graft-versus-leukemia (GVL) effect [1]. For HSCT recipients from HLA-matched donors, the GVL effect can be triggered by minor histocompatibility antigens [2–4], and several studies using sequential flow cytometric analysis with tetramers have clearly demonstrated that minor histocompatibility antigen-specific T lymphocytes increase in frequency in the recipient's blood before and during clinical regression of leukemia [5–10]. On the other hand, for HLA-mismatched HSCT recipients, extremely limited biological studies have demonstrated that the GVL effect can be mediated by mismatched HLA-specific donor T lymphocytes [11].

Allogeneic HSCT is a well-established immunotherapy for leukemia, but, unfortunately, some recipients relapse after transplantation. It is difficult to evaluate the role of individual factors in relapse. Nevertheless, it is reasonable to assume that the selective pressure exerted by donor T lymphocytes can lead to the outgrowth of pre-existing leukemia variants that have lost expression of gene products such as HLA molecules. Some studies have demonstrated loss of the mismatched HLA haplotype in the leukemia blasts of HSCT recipients as a consequence of loss of heterozygosity in chromosome 6 [12–14]. However, the mechanisms involved in leukemia relapse after HLA locus-mismatched HSCT remain largely uninvestigated.

This paper presents a case of selective HLA down-regulation in post-transplant leukemia blasts but not in pre-transplant blasts of a recipient who received bone marrow transplantation from an HLA one locus-mismatched donor. All cytotoxic T lymphocyte (CTL) clones that were isolated from the recipient's blood during acute graft-versus-host disease (GVHD) demonstrated cytotoxicity specific for the mismatched HLA-B molecule, lysed pre-transplant blasts but not post-transplant blasts, and persisted in the patient's blood until leukemia relapse. These results suggest that immune escape of leukemia blasts from CTL pressure toward a certain HLA allele can lead to clinical relapse.

2. Patient, materials and methods

2.1. Patient

A 24-year-old man with primary refractory T lymphoblastic leukemia/lymphoma received allogeneic bone marrow transplantation without ex vivo T lymphocyte depletion from his mother. Because the patient had neither a sibling nor an HLA-matched unrelated donor, his mother was chosen as an alternative donor. PCR sequencing-based typing for HLA alleles of the patient and mother revealed one HLA-B allele mismatch in

* Corresponding author. Fax: +81 52 744 2157.
E-mail address: mmurata@med.nagoya-u.ac.jp (M. Murata).
[1] These authors contributed equally.

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Table 1
HLA types of the patient and donor.

	A	B	C	DRB1	DQB1	DPB1
Patient	1101/2402	5401/5101	0102/–	0901/–	0303/–	0501/–
Donor	1101/2402	5401/5201	0102/1202	0901/1502	0303/0601	0501/–

The mismatched HLA allele in the graft-versus-host direction is underlined.

the graft-versus-host direction (Table 1). The preparative regimen consisted of 180 mg/m^2 melphalan and 12 Gy total body irradiation. GVHD prophylaxis consisted of 0.03 mg/kg tacrolimus and short-term methotrexate. Neutrophil engraftment (neutrophil count $\geqslant 0.5 \times 10^9$/l) was achieved 14 days after transplantation with full donor-type chimera. The patient developed severe acute GVHD involving the skin, gut, and liver on day 46 (maximum stage: skin 3, gut 2, and liver 1; maximum grade: III on day 53), evaluated according to previously published criteria [15]. Acute GVHD was temporarily controlled by additional immunosuppressants, but it was incurable and transitioned to chronic GVHD. On day 261, the patient relapsed with ascites, a hydrocele, and a subpapillary tumor. Leukemia blasts in the ascites fluid were confirmed by cytological examination. Immunosuppressant therapy was required to control GVHD until his death on day 279.

2.2. Cell culture

CTL clones were isolated from a blood sample as described previously [16]. Briefly, peripheral blood mononuclear cells (PBMCs) obtained from the recipient on day 56, when severe acute GVHD developed, were stimulated in vitro with aliquots of γ-irradiated PBMCs that had been obtained from the recipient pre-transplant and cryopreserved. After three weekly stimulations, the CTL clones were isolated from the polyclonal T lymphocyte culture by limiting dilution. The CTLs were expanded by stimulation every 14 days with 30 ng/ml OKT3 monoclonal antibody (Janssen Pharmaceutical), using unrelated allogeneic γ-irradiated (25 Gy) PBMCs and γ-irradiated (75 Gy) EB virus-transformed lymphoblastoid cells (B-LCL) as feeder cells. The culture medium consisted of RPMI-1640-HEPES (Sigma–Aldrich) containing 10% pooled, heat-inactivated human serum, and recombinant human IL-2 (R&D Systems). The T lymphocytes were used in assays 14 days after stimulation or 1 day after thawing of a frozen aliquot. All samples were collected after written informed consent had been obtained. B-LCLs were maintained in RPMI-1640-HEPES with 10% FBS. COS cells were maintained in DMEM (Sigma–Aldrich) with 10% FBS.

2.3. Flow cytometric analysis

Leukemia blasts were incubated at 37 °C for 30 min with anti-HLA-A24/A23 (One lambda), anti-HLA-A11/A1/A26 (One lambda), and anti-HLA-B51/B52/B49/B56 (One lambda) antibodies to detect A24, A11, and B51, respectively, of patient cells followed by incubation at 37 °C for 15 min with fluorescein isothiocyanate-conjugated antimouse IgM (Beckman Coulter). To detect HLA-DR9 of patient cells, leukemia blasts were incubated at 37 °C for 30 min with fluorescein isothiocyanate-conjugated anti-HLA-DR antibody (BD Pharmingen). Antibody to detect HLA-B54 without cross-reaction to B51 was not available. After washing, the cells were analyzed by a BD FACSAria (BD Biosciences). Leukemia blasts were sorted by BD FACSAria with anti-CD7 (BD Biosciences) and anti-CD10 (eBiosciences) antibodies from pre-transplant bone marrow and post-transplant ascites fluid samples. The purities of pre-transplant and post-transplant blasts were ~62% and ~99%, respectively. CTL clones were analyzed using three-color flow cytometry for expression of CD3, CD4, and CD8 using phycoerythrin-cyanin 5.1-conjugated anti-CD3 (Beckman Coulter), phycoerythrin-conjugated anti-CD4 (BD Biosciences), and fluorescein isothiocyanate-conjugated anti-CD8 (BD Biosciences) antibodies.

2.4. Chromium release assay

Leukemia blasts and B-LCLs were used as target cells in a cytotoxicity assay. Leukemia blasts and B-LCLs were labeled for 2 h with ^{51}Cr. After washing, the cells were dispensed at 2×10^3 cells/well into triplicate cultures in 96-well plates and incubated for 4 h at 37 °C with CTL clones at various E:T ratios. Percent-specific lysis was calculated as [(experimental cpm – spontaneous cpm)/(maximum cpm – spontaneous cpm)] ×100.

2.5. Determination of T cell receptor (TCR)-Vβ gene usage and nucleotide sequences

TCR Vβ usage was assessed by RT-PCR using primers covering the entire families of functional TCR Vβ chains [17–19]. Briefly, total RNA was extracted from individual CTL clones, and cDNA was synthesized using SuperScript III RT (Invitrogen). RT-PCR reactions were carried out with the appropriate Vβ sense primers specific for different Vβ families and a primer specific for the constant region of TCR-β. Subsequently, the complementarity determining region 3(CDR3) of each positive PCR product was sequenced with corresponding antisense primer. TCR Vβ gene usage was determined by the international ImMunoGeneTics information system (IMGT) software, IMGT/V-QUEST (http://www.imgt.org/).

2.6. HLA-B cDNA constructs

Total RNA was extracted from the patient and donor B-LCLs and converted into cDNA. Constructs containing the full-length *HLA-B*51:01*, *B*52:01*, and *B*54:01* cDNA were generated from the cDNA by PCR and cloned into the pEAK10 expression vector (Edge BioSystems). Two mutated *HLA-B*51:01* cDNA constructs, in which amino acid at position 63 or 67 was substituted with the corresponding amino acid in *B*52:01*, and two more mutated *HLA-B*51:01* cDNA constructs, in which the amino acid at position 194 or 199 was substituted with the corresponding amino acid in *B*44:03*, were produced using the QuikChange Site-Directed Mutagenesis Kit (Stratagene).

2.7. Transfection of B-LCLs and COS cells with HLA cDNA

B-LCL (5×10^6) were transfected by electroporation (200 V, 500 μFD) in 200 μl of potassium-PBS with the 15 μg of pEAK10 plasmid encoding *HLA-B*51:01* cDNA and selected with puromycin (Edge BioSystems), beginning 48 h after transfection. Three days after selection, they were used as targets in a chromium release assay. COS cells (5×10^3) were plated in individual wells of 96-well flat-bottom plates and transfected with 100 ng of the pEAK10 plasmid encoding *HLA-B*51:01*, *HLA-B*52:01*, *HLA-B*54:01*, or mutated *HLA-B*51:01* cDNA using the FuGENE 6 Transfection Reagent (Roche).

2.8. CTL stimulation assay

COS transfectants (5×10^3) were cocultured with CTL clones (2×10^4) in individual wells of 96-well flat-bottom plates for 24 h at 37 °C, and IFN-γ production was measured in the supernatant using ELISA (Endogen).

2.9. Enzyme-linked immunospot (ELISPOT) assay

T lymphocytes were isolated from recipient's PBMCs by negative depletion using the Pan T Cell Isolation Kit II (Miltenyi Biotec) and used as responder T cells. Responder T cells at a concentration of 2×10^5 per well were plated in individual wells of the 96-well MultiScreen-IP filter plates (Millipore) coated with anti-human interferon (IFN)-γ antibody (5 μg/ml; Mabtech) and tested in triplicate against a total of 2×10^5 stimulator cells: patient B-LCL, donor B-LCL, and *HLA-B*51:01*-transfected donor B-LCL. The plates were incubated for 24 h at 37°C, washed, and incubated with biotinylated anti-human IFN-γ antibody (1 μg/ml; Mabtech) for 2 h at room temperature. After addition of streptavidin (Fitzgerald Industries International) to the wells, the plates were developed with a 3-amino-9-ethylcarbazol substrate kit (Vector Laboratories). Spots were counted using a microscope, and mean numbers were calculated from triplicate wells after subtraction of the number of spots obtained with medium alone.

3. Results

3.1. Selective down-regulation of HLA-B locus in post-transplant leukemia blasts

To determine whether expressions of some HLA loci in post-transplant relapsed leukemia blasts were down-regulated or lost, flow cytometric analysis was performed for HLA-A*24:02, A*11:01, B*51:01, and DR*09:01 using anti-HLA-A24/A23, -HLA-A11/A1/A26, -HLA-B51/B52/B49/B56, and -pan HLA-DR antibodies, respectively. The expression of B*51:01 was down-regulated in post-transplant leukemia blasts compared with that in pre-transplant blasts, whereas expressions of A*24:02, A*11:01, and DR*09:01 were the same or higher in post-transplant blasts than in pre-transplant blasts (Fig. 1). These data led us to question whether B*51:01-selective pressure mediated by donor T lymphocytes was present in the patient post-transplant.

3.2. Isolation of alloreactive CTL clones

Ten CTL clones, termed TK1 to TK10, were isolated from the peripheral blood of the recipient during acute GVHD. In a cytotoxicity assay, all isolated clones lysed recipient B-LCL but failed to lyse donor B-LCL (Fig. 2), demonstrating that all clones were alloreactive. Flow cytometric analysis revealed that all CTL clones

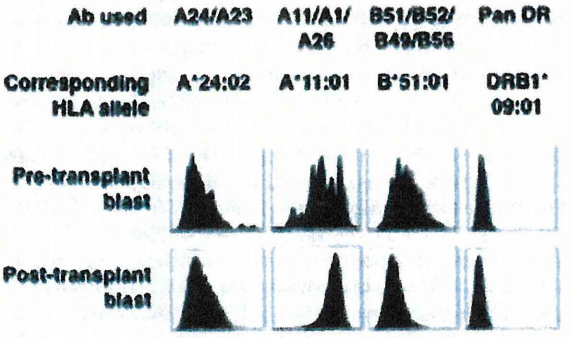

Fig. 1. HLA expression on leukemia blasts. Pre-transplant and post-transplant leukemia blasts were stained with anti-HLA-A24/A23, anti-HLA-A11/A1/A26, anti-HLA-B51/B52/B49/B56, and anti-HLA-pan DR antibodies to detect A*24:02, A*11:01, B*51:01, and DRB1*09:01, respectively. Data are representative of four experiments.

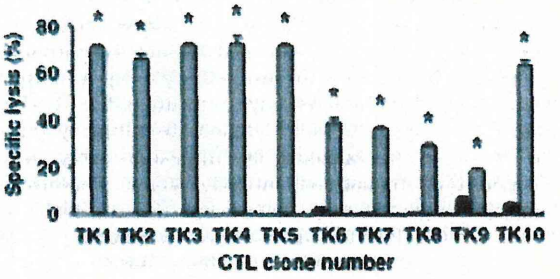

Fig. 2. Cytotoxicities of CTL clones against B-LCLs. B-LCLs that originated from the recipient (gray) and the donor (black) were used as targets for CTL clones. Specific lysis is shown as the mean and SD of triplicate cultures at an E:T ratio of 10:1. *Significant difference ($p < 0.0001$: Student's *t*-test) in the lysis of recipient B-LCL compared with donor B-LCL. Data are representative of three experiments.

Table 2
Clonotypes of isolated CTL clones.

CTL	TCR Vβ	Nucleotide and deduced amino acid sequences of complementarity determining region 3																		
TK1	Vβ6.5	GCC	AGC	AGT	CCC	GGG	ACT	AGC	GGA	ACC	TAC	GAG	CAG	TAC	TTC					
		A	S	S	P	G	T	S	G	T	Y	E	Q	Y	F					
TK2	Vβ20	AGT	CAG	GGG	CCG	GCG	GTT	ACC	GGG	GAG	CTG	TTT	TTT							
		S	Q	G	P	A	V	T	G	E	L	F	F							
TK3	Vβ20	AGT	CAG	GGG	CCG	GCG	GTT	ACC	GGG	GAG	CTG	TTT	TTT							
		S	Q	G	P	A	V	T	G	E	L	F	F							
TK4	Vβ19*1	GCC	AGT	ACT	TGG	GGT	TAC	CCA	CAG	GGG	CCC	GGT	GCG	GAT	ACC	GGG	GAG	CTG	TTT	TTT
		A	S	T	W	G	Y	P	Q	G	P	G	A	D	T	G	E	L	F	F
TK5	Vβ19*1	GCC	AGT	ACT	TGG	GGT	TAC	CCA	CAG	GGG	CCC	GGT	GCG	GAT	ACC	GGG	GAG	CTG	TTT	TTT
		A	S	T	W	G	Y	P	Q	G	P	G	A	D	T	G	E	L	F	F
TK6	Vβ12	GCC	AGC	AGT	TTA	GCT	AGC	GGG	AGG	GCC	TCC	CAT	GAG	CAG	TTC	TTC				
		A	S	S	L	A	S	G	R	A	S	H	E	Q	F	F				
TK7	Vβ12	GCC	AGC	AGT	TTA	GCT	AGC	GGG	AGG	GCC	TCC	CAT	GAG	CAG	TTC	TTC				
		A	S	S	L	A	S	G	R	A	S	H	E	Q	F	F				
TK8	ND																			
TK9	Vβ12	GCC	AGC	AGT	TTA	GCT	AGC	GGG	AGG	GCC	TCC	CAT	GAG	CAG	TTC	TTC				
		A	S	S	L	A	S	G	R	A	S	H	E	Q	F	F				
TK10	Vβ2	GCC	AGC	AGT	GAC	TCT	ATC	GCG	GAT	GAG	CAG	TTC	TTC							
		A	S	S	D	S	I	A	D	E	Q	F	F							

ND, not detected.

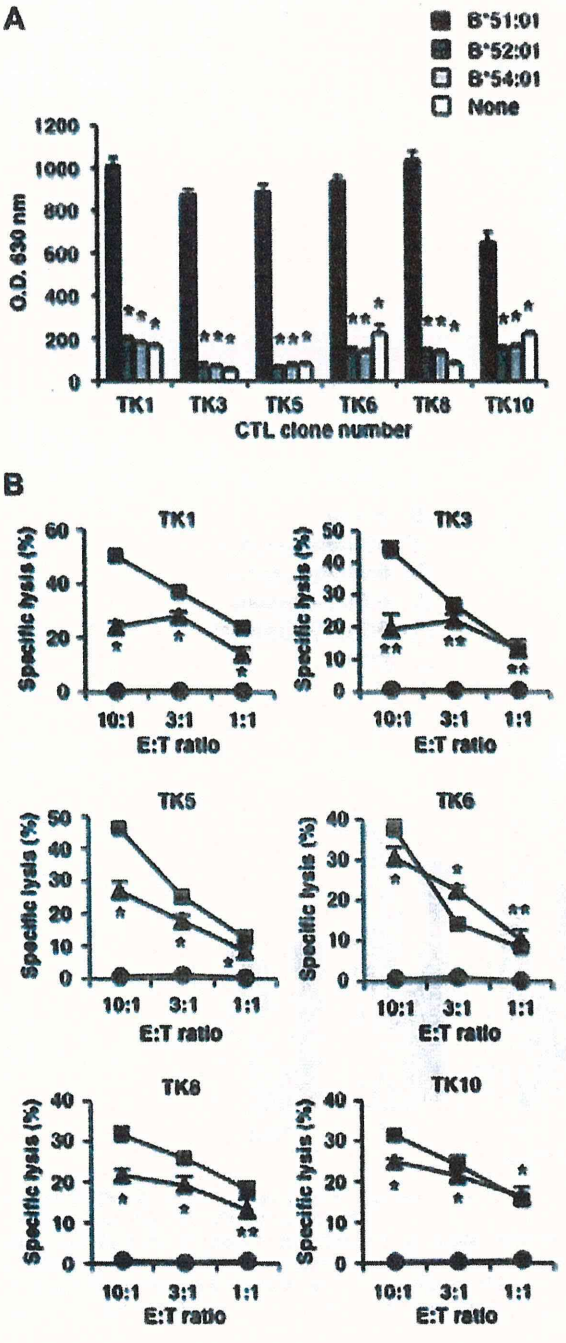

Fig. 3. Recognition of the HLA-B*51:01 molecule by CTLs. (A) COS cells were transfected with a plasmid encoding *B*51:01* cDNA, *B*52:01* cDNA, *B*54:01* cDNA, or no cDNA cocultured with CTL clones, and IFN-γ production was measured in the supernatant using ELISA. Data are the means and SD of triplicate determinations. *Significant difference ($p < 0.01$: Student's *t*-test) in the IFN-γ production stimulated by *B*52:01* cDNA, *B*54:01* cDNA or no cDNA compared with *B*51:01* cDNA. Data are representative of three experiments. (B) Recipient B-LCL (square), donor B-LCL (circle), and donor B-LCL transfected with *HLA-B*51:01* cDNA (triangle) were used as targets for CTL clones. Specific lysis is shown as the mean and SD of triplicate cultures at various E:T ratios. Significant difference (*$p < 0.01$; **$p < 0.05$) in the lysis of *B*51:01*-transfected donor B-LCL compared with donor B-LCL (negative control). Data are representative of three experiments.

were CD3+/CD4−/CD8+ (data not shown). The nucleotide sequences of the uniquely rearranged *TCR Vβ* gene of each clone were determined by direct DNA sequencing of the amplified PCR products of TCR (Table 2). The TK2 and TK3 clones had the same nucleotide sequences in the CDR3 regions of their TCR Vβ20, suggesting that these CTLs originated from a single clone. Similarly, TK4 and TK5, as well as TK6, TK7, and TK9, had the same nucleotide sequences in the CDR3 regions of their TCR Vβ19*1 and Vβ12, respectively, suggesting that each group also originated from a single clone. Thus, the 10 isolated alloreactive CTL clones appeared to have been derived from six independent clones.

3.3. CTL clones recognized the HLA-B*51:01 molecule

To evaluate the possibility that isolated CTL clones recognize the HLA-B*51:01 molecule, COS cells were first transfected with an *HLA-B*51:01*, *–B*52:01*, or *–B*54:01* cDNA construct, COS transfectants were cocultured with six independent CTL clones, and then the production of IFN-γ in the supernatant was measured. The COS cells transfected with *HLA-B*51:01* clearly stimulated IFN-γ production by six independent CTL clones, whereas neither *B*52:01* nor *B*54:01* stimulated them (Fig. 3A). Then, donor B-LCL were transfected with an *HLA-B*51:01* cDNA construct and used as target cells in a cytotoxicity assay. The donor B-LCL transfected with *HLA-B*51:01* were lysed by six CTL clones (Fig. 3B), indicating that all clones recognized the mismatched HLA-B*51:01 molecule as an alloantigen. On the other hand, these data suggest that the CTL response toward the HLA-B*51:01 molecule accounted for the majority of the recipient's CTL alloresponse during acute GVHD.

3.4. Recognition of HLA molecules by CTL clones was peptide-dependent

Various forms of T lymphocyte recognition of the allogeneic major histocompatibility antigen, ranging from peptide-dependent to peptide-independent, have been demonstrated [20]. To confirm peptide dependency in CTL recognition, examinations were focused on the difference in the amino acid sequences of the recipient B*51:01 and the donor B*52:01. They differed in two amino acids at positions 63 and 67 (Fig. 4A), which constitute peptide binding pockets A and/or B [21,22]. In particular, B-pocket has a critical role in peptide binding to HLA-B*51:01 molecules [23], and substitution of a single amino acid constituting peptide binding pocket can affect peptide binding [24]. Two mutated *B*51:01* cDNA constructs, *B*51:01-Asn63Glu* and *B*51:01-Phe67Ser*, in which individual amino acids were substituted with the corresponding amino acid in B*52:01 (Fig. 4A), were generated, as well as two more mutated *B*51:01* cDNA constructs, *B*51:01-Val194Ile* and *B*51:01-Ala199Val*, in which individual amino acids exist in *B*44:02* and other B alleles and localize outside the positions constituting peptide binding pockets. COS cells were then transfected with each wild or mutated cDNA construct and examined in the CTL stimulation assay. IFN-γ production of the TK3 clone was significantly decreased when stimulated by the *B*51:01-Phe67Ser* mutant in comparison with the wild-type *B*51:01* construct (Fig. 4B). IFN-γ production of all other CTL clones, TK1, TK5, TK6, TK8, and TK10, was significantly decreased when stimulated by *B*51:01-Asn63Glu* and *B*51:01-Phe67Ser* mutants in comparison with the wild-type *B*51:01* construct (Fig. 4B). However, both *B*51:01-Val194Ile* and *B*51:01-Ala199Val* mutants stimulated all CTL clones to the same degree as the wild-type *B*51:01* construct. Thus, these data suggest that recognition of the HLA-B*51:01 molecule by CTL clones was peptide-dependent.

Furthermore, CTL clones should recognize certain peptides other than leukemia antigens, presented by HLA-B*51:01 molecules, because *B*51:01*-transfected COS cells, which are derived from monkey kidney cells, stimulated IFN-γ production of CTLs