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Outcome of 125 Children with Chronic Myelogenous Leukemia Who Received Transplants from Unrelated Donors: The Japan Marrow Donor Program

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Because of a small number of patients, only a few studies have addressed the outcome of bone marrow transplantation (BMT) in children with Philadelphia chromosome–positive (Ph+) chronic myelogenous leukemia (CML), who receive graft from a volunteer-unrelated donor (VUD), especially after practical application of imatinib mesylate. The outcomes of BMT from a VUD in 125 children with Ph+ CML were retrospectively reviewed. Patients were identified through the Japan Marrow Donor Program as having undergone BMT between 1993 and 2005 and were aged 1-19 years at the time of transplant (median age, 14 years). The probabilities of 5-year overall survival (OS) and leukemia-free survival (LFS) were 59.3% and 55.5%, respectively. Multivariate analysis identified the following unfavorable survival factors: infused total nucleated cell dose $< 3.14 \times 10^6$ /kg (relative risk [RR] = 2.43; 95% confidence interval [CI] = 1.33-4.44; $P = .004$), advanced phase (RR = 2.43; 95% CI = 1.37-4.31; $P = .004$), and no major cytogenetic response (MCyR) at the time of BMT (RR = 6.55; 95% CI = 1.98-21.6; $P = .002$). Of the 17 patients treated with imatinib, 15 (88%) achieved MCyR at the time of BMT, and this group had an excellent 5-year OS of 81.9%. Disease phase, infused total nucleated cell dose, and cytogenetic response were independent risk factors for survival of unrelated BMT. These findings provide important information for assessing the indications for and improving outcome in unrelated BMT for the treatment of pediatric CML.

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INTRODUCTION

Philadelphia-positive (Ph+) chronic myelogenous leukemia (CML) is a rare disease in children, accounting for only 3%-5% of all pediatric leukemia, with a incidence of <1 in 100,000 children [1]. Allogeneic hematopoietic stem cell transplantation (HSCT) is the only proved curative treatment for children with Ph+ CML. Reported event-free survival (EFS) in children with Ph+ CML who underwent transplantation in the

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chronic phase with a matched related donor is 60%-75% [2-4]; however, this approach is limited by the availability of HLA-matched family donors. The majority of children who lack an HLA-matched donor receive a transplant from an alternative donor, such as a volunteer-unrelated donor (VUD). EFS is less favorable in this setting, ranging from 30% to 55% [3-5].

Since the introduction of the novel tyrosine kinase inhibitor imatinib mesylate, the treatment for Ph+ CML has been completely revised [6]. Imatinib can induce complete hematologic and cytogenetic remission in the majority of patients, and follow-up data on patients treated only with imatinib indicate that complete cytogenetic and major molecular responses are durable, while drug toxicity is low [7]. The number of transplantations for Ph+ CML has declined rapidly [8]. But, despite significant cytogenetic and molecular responses, there is no evidence that imatinib is curative, and imatinib's long-term side effects remain to be determined. Some patients have successfully stopped imatinib without recurrence, but some who were polymerase chain reaction (PCR)-negative for a period stopped and then experienced recurrence [9,10]. Stopping imatinib may be possible, but effective strategies have yet to be developed.

This is particularly important for pediatric patients, in whom the goal is cure of the disease rather than palliation, and for whom long-term survival is particularly anticipated. The presence of molecular disease and the emergence of resistant clones in patients treated with imatinib suggest the need for caution with regard to abandoning curative therapy by SCT. The need for information on the current status of SCT for Ph+ CML and up-to-date results when considering the treatment of children with Ph+ CML, even in the imatinib era, is evident; however, few studies have specifically analyzed outcomes of SCT in children with Ph+ CML [2-5]. The aim of the present study was to analyze data from 125 children with Ph+ CML who underwent bone marrow transplantation (BMT) from a VUD and identify factors influencing outcome.

PATIENTS AND METHODS

Patients

A retrospective analysis was conducted on behalf of the Japan Marrow Donor Program (JMDP) and the Japanese Pediatric Leukemia/Lymphoma Study Group (JPLSG) CML Committee. Data were collected from 125 children (age at transplantation < 20 years) whose donors were identified through the JMDP and who underwent allogeneic BMT from a VUD for Ph+ CML between 1993 and 2005. Table 1 summarizes the patient, donor, and transplant characteristics. Patient characteristics in the first chronic phase (CP1) and in the advanced phase are described separately. All patients or their guardians gave written informed consent for transplantation and submission of data to the JMDP for further research. This study

Table 1. Patient, Donor, and Transplant Characteristics

	CP1 (n = 88)	Advanced Phase (n = 37)	Total (n = 125)
Year of transplantation			
1993-1998	45	22	67
1999-2005	43	15	58
Stage of CML at BMT			
CP1	88	0	88
CP2	0	12	12
CP3	0	1	1
Advance phase	0	11	11
Blast crisis	0	13	13
Cytogenetic response at BMT			
With MCyR	29	4	33
Without MCyR	39	25	64
Unknown	20	8	28
Pretransplantation therapy with IFN-α			
No	22	8	30
Yes	66	29	95
Pretransplantation therapy with imatinib			
No	72	36	108
Yes	16	1	17
Recipient sex, M/F	56/32	25/12	81/44
Donor–recipient sex			
Female donor to male recipient	20	10	30
Other	68	27	95
Median age at BMT, years (range)	13 (1-19)	17 (2-19)	14 (1-19)
Median time from diagnosis to transplantation, months (range)	14 (2-111)	19 (5-103)	14 (2-111)
Patient CMV antibody			
Negative	25	14	39
Positive	54	21	75
Unknown	9	2	11
ABO mismatch			
Match	41	15	56
Major mismatch	29	11	40
Minor mismatch	17	9	26
Unknown	1	2	3
Recipient–donor HLA DNA typing			
Match (10/10)	33	8	41
1 alleles mismatch	9	5	14
2 alleles mismatch	19	9	28
3 alleles mismatch	8	3	11
4 alleles mismatch	2	2	4
6 alleles mismatch	0	1	1
Unknown	17	9	26
Conditioning regimen			
TBI regimen	66	30	96
Non-TBI regimen	22	7	29
GVHD prophylaxis			
CsA + MTX	59	22	81
Tacrolimus + MTX	28	15	43
MTX alone	1	0	1
Administration of ATG			
No	76	34	110
Yes	12	3	15
Median infused total nucleated cell dose, $\times 10^6$ /kg (range)	315 (27-880)	298.5 (29-750)	314 (27-880)

ATG indicates antithymocyte globulin; BMT, bone marrow transplantation; CML, chronic myelogenous leukemia; CP, chronic phase; CMV, cytomegalovirus; CsA, cyclosporine; IFN, interferon; GVHD, graft-versus-host disease; MCyR, major cytogenetic response; MTX, methotrexate; TBI, total body irradiation.

was approved by the Data Management Committee of the JMDP and by the Ethical Committee of Nagoya University Graduate School of Medicine.

The 125 children in the study included 81 boys (65%) and 44 girls (35%). The median age at the time of BMT was 14 years (range, 1-19 years). Disease phase at the time of transplantation was defined according to International Bone Marrow Transplant Registry (IBMTR) criteria [11]. Eighty-eight patients (70%) underwent transplantation in CP1. Of the 37 children who underwent transplantation in an advanced phase of CML, 12 were in CP2, 1 was in CP3, 11 were in the accelerated phase (AP), and 13 were in blast crisis (BC). Cytogenetic response data at the time of BMT were available for 97 patients (78%), of whom 68 were in CP1 and 29 were in an advanced phase. Major cytogenetic response (MCyR; $\leq$ 35% Ph+ cells) was achieved in 33 patients (29 patients in CP1 and 4 patients in CP2). Ninety-five recipients (76%) were given interferon (IFN)-α, and 17 (14%) were given imatinib before transplantation. The patients treated with imatinib proceeded to BMT regardless of their response, according to each institutes' therapeutic strategy. The median interval from diagnosis to transplantation was 14 months (range, 2-111 months). Fifty-seven patients (46%) underwent transplantation within 12 months, and 68 (54%) did so after 12 months. Imatinib began to be used in Japan in 1999, and its use was approved by the Japanese Health and Welfare Ministry in 2002. In our cohort, 17 patients (16 in CP1, 1 in AP) received imatinib before transplantation.

Transplantation Procedures and Recipient–Donor HLA Matching

All 125 recipients received a BM graft from a VUD identified through the JMDP. Various preconditioning regimens were used by individual centers. Of the 125 recipients, 96 (77%) received a preparative regimen with total body irradiation (TBI). Fifteen recipients (12%) received antithymocyte globulin (ATG). Cyclosporine A (CsA)–based GVHD prophylaxis was used in 81 patients (65%); tacrolimus-based prophylaxis, in 43 (34%). One patient received only methotrexate (MTX) as GVHD prophylaxis. HLA-matching data based on high-resolution DNA typing for HLA-A, -B, -C, -DRB1, and -DQB1 antigens were available in 99 patients (79%). Of these 99 patients, 41 (41%) were fully matched at 10/10 alleles, 14 (14%) were mismatched at 1 HLA allele, 28 (28%) were mismatched at 2 HLA alleles, and 16 (16%) were mismatched at more than 3 HLA alleles.

Definitions, Data Collection, and Statistical Analysis

The outcomes were analyzed on the basis of engraftment, grade II-IV acute and chronic GVHD (aGVHD, cGVHD), treatment-related mortality (TRM), relapse, overall survival (OS), and leukemia-free survival (LFS). The date of engraftment was defined as the first of 3 consecutive days with a neutrophil count exceeding 0.5×10^9 /L. aGVHD and cGVHD were classified according to published criteria [12]. Only patients surviving for >100 days after transplantation were considered eligible for evaluation of cGVHD. Relapse of CML was defined by hematologic or cytogenetic evidence of disease. (Data on molecular evidence of relapse were not available.) Transplantation data were collected using standardized forms provided by the JMDP. After transplantation, patient baseline information and follow-up reports were submitted at 100 days, 6 months, 1 year, and annually thereafter.

Comparisons between groups were performed using Fisher's exact test for categorical variables and the Mann-Whitney U test for continuous variables. Survival and time to events were calculated from the date of transplantation. OS and LFS were estimated by the Kaplan-Meier method and compared using the log-rank test. Cumulative incidence curves were created for TRM. The Cox proportional hazard model was used to obtain the estimates and the 95% confidence interval (CI) of the relative risk (RR) for predictive factors and to evaluate predictive factors for TRM, LFS, and OS in a multivariate analysis. The following variables were evaluated: patient age at the time of BMT ($\geq$ 15/< 15 years), patient sex, sex mismatch, year of transplantation (1993-1998/1999-2005), period from diagnosis to transplantation ($\geq$ 12 months/< 12 months), infused total nucleated cell dose ($\geq 314 \times 10^6$/kg/< 314×10^6/kg), TBI-containing regimen (yes/no), use of ATG (yes/no), GVHD prophylaxis (CsA + MTX $\pm$ steroids/FK $\pm$ MTX), full HLA matching (yes/no), disease phase at the time of BMT (CP1/advanced phase), MCyR at the time of BMT (yes/no), ABO mismatch (match/mismatch), recipient cytomegalovirus (CMV) antibody (negative/positive), history of interferon therapy (yes/no), and history of imatinib therapy (yes/no). Variables with more than 2 categories were dichotomized for the final multivariate model. The cutoff points of the variables were chosen to make optimal use of the information, with the proviso that smaller groups contained at least 20% of the patients. The cutoff points of continuous variables were chosen from the 25th, 50th, and 75th percentiles; consequently, the median of continuous variables was dichotomized as follows: age ($\geq$15/< 15 years), year of transplantation (1993-1998/1999-2005), and infused total nucleated cell dose ($\geq 314 \times 10^6$/kg/< 314×10^6/kg). SPSS version 15.0 (SPSS Inc, Chicago, IL) was used for all statistical calculations except estimation of the cumulative incidence, which was performed using Stata version 10.0 (StataCorp, College Station, TX).

Table 2. Patient Clinical Outcomes

	CP1 (n = 88)	Advanced Phase (n = 37)	Total (n = 125)	P Value
Engraftment				.336
Yes/No	85 / 3	34 / 3	119 / 6	
Acute GVHD				.186
None	21	11	32	
Grade I	34	9	43	
Grade II	18	5	23	
Grade III	11	7	18	
Grade IV	4	5	9	
Chronic GVHD				.393
None	49	25	74	
Limited	15	6	21	
Extensive	24	6	30	
5-year TRM (95% CI)	28.3% (23.4-33.2)	56.5% (48.0-65.0)	36.5% (32.5-40.5)	.002
5-year relapse rate (95% CI)	11.8% (8.1-15.5)	29.0% (18.7-39.3)	15.4% (11.7-19.1)	.098
5-year LFS (95% CI)	65.2% (60.0-70.4)	32.4% (24.7-40.1)	55.5% (51.0-60.0)	.001
5-year OS (95% CI)	70.7% (65.7-75.7)	32.4% (24.7-40.1)	59.3% (54.8-63.8)	<.001

GVHD indicates graft-versus-host disease; LFS, leukemia-free survival; OS, overall survival; TRM, treatment-related mortality.

RESULTS

Engraftment

A total of 119 recipients (95%) were successfully engrafted. Neutrophil engraftment occurred at a median of 18 days after BMT (range, 11-37 days). Six patients (5%) experienced primary graft failure (Table 2), all of whom died.

aGVHD and cGVHD

Grade II-IV aGVHD occurred in 50 patients (40.7%; 95% CI = 36.3%-45.1%), and grade III-IV aGVHD occurred in 27 patients (22.6%; 95% CI = 16.1%-31.2%). Fifty-one patients (50.1%; 95% CI = 45.0%-55.2%) developed cGVHD (extensive type, n = 30; limited type, n = 21).

Relapse

Seventeen patients (11 recipients in CP1 and 6 in an advanced phase) experienced a relapse. The 5-year cumulative incidence of relapse was 19.7% (95% CI = 15.1%-24.3%). The median time for occurrence of relapse for the entire study cohort was 7 months (range, 1-97 months).

Survival

LFS

The 5-year LFS rate was 55.5% (95% CI = 51.0%-60.0%) for the entire cohort (Figure1). The LFS rate was significantly higher in children undergoing BMT in CP1 (65.2%; 95% CI = 60.0%-70.4%) than those undergoing BMT in an advanced phase (32.4%; 95% CI = 28.7%-36.1%; $P = .001$) (Table 2).

On univariate analysis, the following factors were significantly associated with LFS: age at the time of BMT ($P = .047$), infused total nucleated cell dose ($P = .002$), disease phase ($P = .002$), and cytogenetic response at the time of BMT ($P = .001$). Multivariate analysis also identified infused total nucleated cell dose (RR = 2.320; 95% CI = 1.326-4.061; $P = .003$), disease phase (RR = 2.051; 95% CI = 1.187-3.545; $P = .010$), and cytogenetic response at the time of BMT (RR = 2.890; 95% CI = 1.264-610); $P = .012$) as independent risk factors for LFS.

OS

The 5-year OS rate was 59.3% (95% CI = 54.8%-63.8%) for the entire cohort (Figure 1). The OS rate was significantly higher in the children undergoing BMT in CP1 (70.7%; 95% CI = 65.7%-75.7%) than in those undergoing BMT in an advanced phase (32.4%; 95% CI = 24.7%-40.1%; $P < .001$) (Table 2).

On univariate analysis, the following risk factors were significantly associated with OS: age at the time of BMT ($P = .037$), interval between diagnosis and

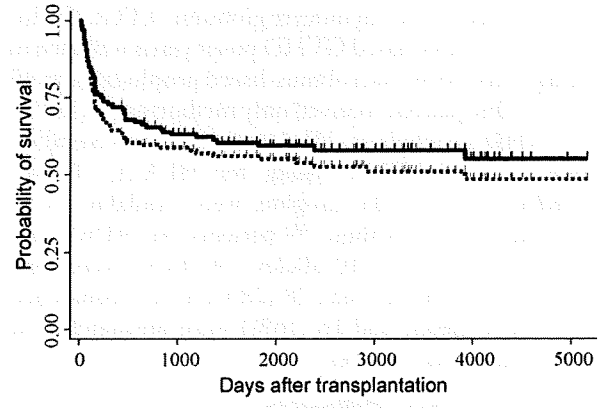

Figure 1. OS and LFS in children with Ph+ CML. In Kaplan-Meier curves graph, solid line shows the probabilities of OS (5-year OS = 59.3%; 95% CI = 54.8%-63.8%) and the dotted line shows that of LFS (5-year LFS = 55.5%; 95% CI = 51.0%-60.0%).

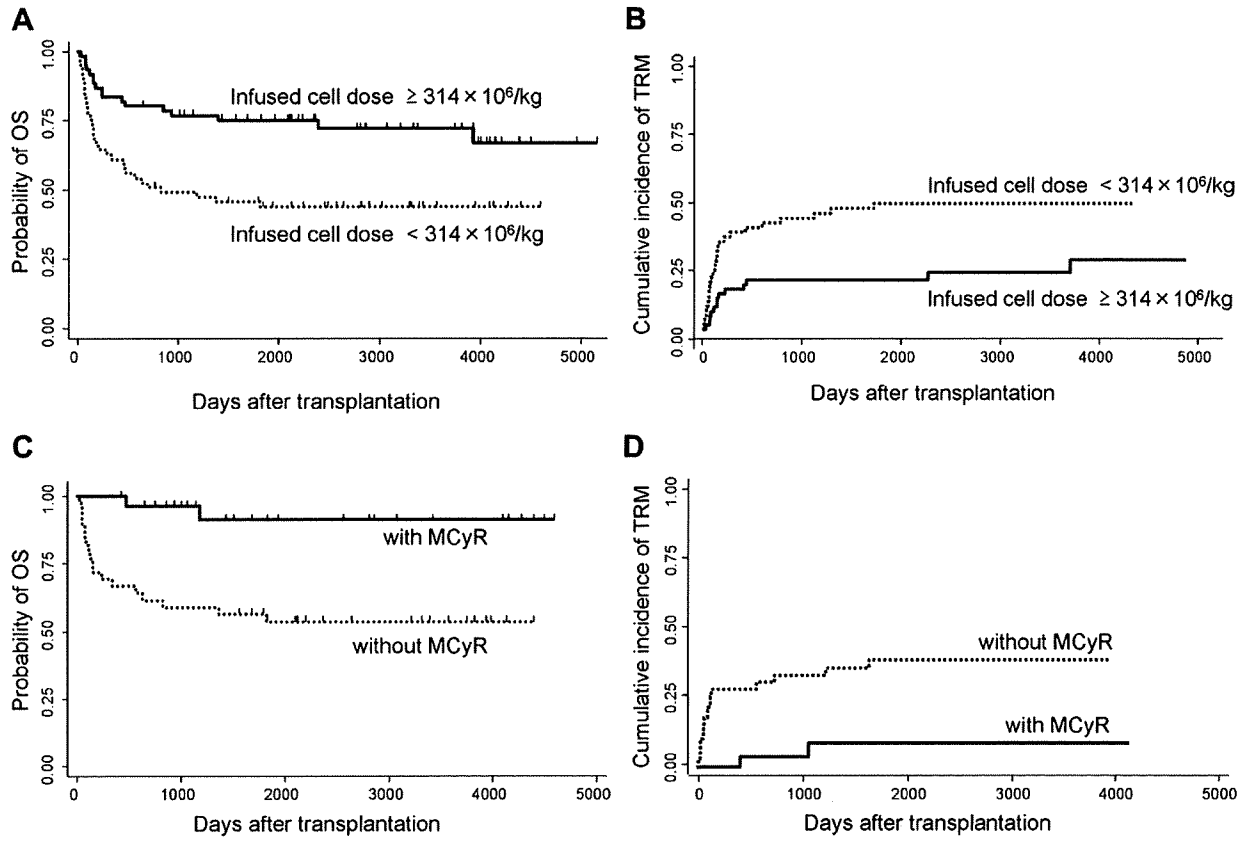

Figure 2. A and B, Relationship among infused total nucleated cell dose, OS (A), and TRM (B) in children with Ph+ CML. In the entire cohort, OS was significantly higher for children who received a higher infused total nucleated cell dose than those who received a lower dose (≥ 314 × 10^6/kg vs < 314 × 10^6/kg; $P = .001$). TRM was significantly higher for children who received a lower cell dose than for those who received a higher cell dose (≥ 314 × 10^6/kg vs < 314 × 10^6/kg; $P = .003$). Solid lines show the probabilities of OS and TRM for children who received a higher infused total nucleated cell dose and the dotted lines show the probabilities for those who received a lower infused total nucleated cell dose. C and D, OS (C) and TRM (D) of Ph+ CML children in CP1 with or without an MCyR. OS was significantly higher for children who achieved MCyR at the time of BMT (n = 29) than for those who did not (n = 39) (OS; $P < .001$) (C). TRM was also significantly higher for children who did not achieve MCyR ($P = .005$) (D). The solid lines show the probabilities of OS and TRM for children with MCyR at the time of BMT, and the dotted lines show the probabilities for those without.

BMT ($P = .042$), infused total nucleated cell dose ($P = .002$), disease status ($P < .001$), and cytogenetic response at the time of BMT ($P = .002$). A history of imatinib therapy before BMT marginally affected OS ($P = .099$). Multivariate analysis identified infused total nucleated cell dose (RR = 2.426; 95% CI = 1.326-4.441; $P = .001$) (Figure 2A), disease status (RR = 2.427; 95% CI = 1.368-4.305; $P = .002$), and cytogenetic response at the time of BMT (RR = 6.547; 95% CI = 1.982-21.629; $P = .002$) (Figure 2C) as independent risk factors for OS (Table 3).

Causes of Death

Fifty-two patients (42%) died after BMT from a VUD (Table 4). The day-100 mortality rate was 15.2 % (95% CI = 12.0%-18.4%). The main cause of death was transplantation-related complications, from which 46 patients (37%) died between day 8 and 10 years (median, 4 months) after transplantation. These included 18 transplantation-related deaths occurring before day 100 after transplantation. Death was associated with treatment-resistant GVHD in 14 patients (9 with aGVHD and 5 with cGVHD). Infection was the cause of death in 12 patients. Six patients died from recurrent CML between 3 and 28 months (median, 13 months) after transplantation.

Univariate analysis revealed that infused cell dose ($P = .013$), disease phase ($P = .006$), and cytogenetic response at the time of BMT ($P = .001$) were significant risk factors for TRM. The interval between diagnosis to BMT ($P = .083$) and HLA mismatch ($P = .087$) were marginally associated with TRM. In the multivariate model, infused cell dose (RR = 2.347; 95% CI = 1.195-4.610; $P = .013$) (Figure 2B) and cytogenetic response at the time of BMT (RR = 9.055; 95% CI = 2.151-38.127; $P = .003$) (Figure 2D) were independent risk factors for TRM (Table 3).

Effects of HLA Compatibility

The influence of HLA compatibility between recipient and donor on aGVHD, TRM, and OS was assessed by univariate analysis. aGVHD (grade II-IV)

Table 3. Risk Factors for TRM and OS on Multivariate Analysis

Covariates	RR (95% CI)	P value
TRM		
Infused cell dose		
$\geq 314 \times 10^6$/kg	(1)	
$< 314 \times 10^6$/kg	2.347 (1.195-4.610)	.013
Cytogenetic response at BMT		
With MCyR	(1)	
Without MCyR	9.055 (2.151-38.127)	.003
OS		
Infused total nucleated cell dose		
$\geq 314 \times 10^6$/kg	(1)	
$< 314 \times 10^6$/kg	2.426 (1.326-4.441)	.004
Disease phase at BMT		
CP1	(1)	
Advanced phase	2.427 (1.368-4.305)	.002
Cytogenetic response at BMT		
With MCyR	(1)	
Without MCyR	6.547 (1.982-21.629)	.002

BMT indicates bone marrow transplantation; MCyR, major cytogenetic response; OS, overall survival; TRM, treatment-related mortality.

was less frequent in patients with fully matched donors than in those with mismatched donors (RR = 2.044; 95% CI = 1.055-3.961; P = .034). TRM (RR = 1.902; 95% CI = 0.894-4.045; P = .095) and OS (RR = 1.572; 95% CI = 0.817-3.027; P = .176) tended to be worse in mismatched transplantation, but the difference was not statistically significant. In the analysis of each single allele mismatch, only the HLA-A allele mismatch significantly affected OS (RR = 2.837; 95% CI = 1.347-5.977; P = .006). HLA-C mismatch marginally affected OS (RR = 1.639; 95% CI = 0.945-2.843; P = .078), whereas HLA-B, -DRB1, and -DQB1 mismatch were not significant. On multivariate analysis, HLA compatibility was not identified as an independent risk factor for acute GVHD, TRM, or OS.

Table 4. Causes of Death

	CP1 (n = 88)	Advanced Phase (n = 37)	Total (n = 125)
TRM	26	20	46
Infections			
Bacterial	4	1	5
Fungal	1	0	1
Viral	3	1	4
Pneumocystis jirovecii	1	0	1
Unknown	0	1	1
Rejection	0	1	1
Acute GVHD	5	4	9
Chronic GVHD	4	1	5
Idiopathic interstitial pneumonitis	6	4	10
Cardiac failure	0	1	1
Respiratory failure	0	1	1
Renal failure	1	1	2
Hemorrhage	0	2	2
Secondary malignancy	1	0	1
Unknown	0	2	2
Relapse	1	5	6

CP indicates chronic phase; GVHD, graft-versus-host disease; TRM, treatment-related mortality.

Effect of Cytogenetic Response at Transplantation

Cytogenetic response data were available in 68 of 88 patients (77%) who underwent transplantation in CP1. Sixteen patients received imatinib, 35 received IFN-α, and 3 received neither imatinib nor IFN-α. MCyR at the time of BMT was achieved in 15 of the 16 patients (94%) treated with imatinib and in 14 of the 35 patients (40%) treated with IFN-α.

Patients with MCyR at the time of BMT (n = 29) had significantly better OS and LFS than those without MCyR (n = 39): 5-year OS = 91.4%, 95% CI = 85.4%-97.4% versus 53.4% and 45.3%-61.5% (P = .001); 5-year LFS = 81.0 %, 95% CI = 73.2%-88.8% versus 50.9% and 42.8%-59.0% (P = .02) (Figure 2C). Although no significant difference in relapse rate was seen between the 2 patient groups (P = .91), TRM was significantly lower in those who achieved MCyR at the time of BMT (n = 29) than in those who did not (n = 39): 5-year TRM = 9.6 %, 95% CI = 3.0%-16.2% vs 41.0% and 32.7%-49.3% (P = .005) (Figure 2D).

Effect of Pre-BMT Imatinib Therapy

In this cohort, 17 patients received imatinib before transplantation, and 15 of them (88.2%) achieved MCyR in CP1 before transplantation. This percentage was significantly higher than that in the patients who did not receive imatinib (88.2% vs 22.2%; $P < .01$). A history of imatinib therapy had a positive effect on survival (5-year OS = 81.9 %, 95% CI = 72.4%-91.4% vs 56.4% and 51.6%-61.2%; P = .086), but this effect was not statistically significant.

DISCUSSION

Because of the small number of patients, to date only a few studies have addressed the outcome of children with Ph+ CML undergoing BMT with a VUD [3-5]. The number of patients in the present study is comparable to that of the largest previous study, which included 132 children with CML undergoing BMT from a VUD [4]. Furthermore, unlike that previous study, our data set contains detailed information on infused total nucleated cell dose, high-resolution HLA compatibility, and cytogenetic response at the time of BMT. Until now, these variables have not been evaluated in a pediatric CML population.

In clinical settings [13-15], as well as in animal models [16,17], larger cell dose is recognized as an important predictor of a favorable outcome for allogeneic BMT. When an adult patient with CML receives a transplant from a VUD, a lower infused total nucleated cell dose is associated with an increased incidence of TRM [18]. Our findings also demonstrate an association between lower infused total nucleated cell dose

and lower OS and LFS and a higher incidence of TRM. These correlations are independent of recipients' age. Moreover, all 6 patients who experienced graft failure were in the lower infused total nucleated cell dose group. Based on our findings, we recommend BM harvest teams attempt to collect a higher number of nucleated cells for infusion in CML patients undergoing BMT from a VUD.

Cytogenetic response to previous treatment with IFN-α [19] and imatinib [20] has been reported to be predictive for survival after allogeneic SCT in Ph+ CML. In the multivariate model of our entire cohort, MCyR at the time of BMT was an independent predictive factor for transplantation outcome. Furthermore, subgroup analysis of the patients in CP1 confirmed that the lower TRM rate in patients with MCyR at the time of BMT contributed to a better survival rate (Figure 2C), suggesting that MCyR is important for better transplantation outcome in CP1 CML as well. Recently, the Center for International Blood and Bone Marrow Transplant Research reported a significantly lower TRM and a better OS in imatinib-treated patients undergoing allogeneic SCT [21]. In our cohort, the imatinib-treated patients tended to have a higher OS ($P = .086$), but the difference was not statistically significant; however, our imatinib-treated group was small (17 of 125 patients), which may have reduced the statistical power.

We have now multiple treatment modalities for pediatric CML, including allogeneic SCT, imatinib, and, more recently, second-generation tyrosine kinase inhibitors. Although only few small studies have analyzed the data on pediatric imatinib monotherapy [22,23], those studies have reported comparable results to adult large clinical trials [24-26]. Growth disturbance as a side effect of imatinib in a pediatric CML patient was reported recently [27]; this effect could be a serious drawback to long-term imatinib therapy in the future. Of course, allogeneic SCT also has potential long-term sequelae, including growth retardation. We are currently planning a study comparing the long-term outcomes and complications of therapy with tyrosine kinase inhibitors and allogeneic SCT in the imatinib era.

In summary, disease phase, infused total nucleated cell dose, and cytogenetic response at the time of BMT were found to be independent risk factors for OS, LFS, and TRM in BMT from a VUD for the treatment of pediatric CML. These results provide important information for evaluating indications and improving outcome in children with CML undergoing unrelated BMT.

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REFERENCES

1. Altman AJ. Chronic leukemias of childhood. *Pediatr Clin North Am.* 1988;35:765-787.
2. Creutzig U, Ritter J, Zimmermann M, Klingebiel T. Prognosis of children with chronic myeloid leukemia: a retrospective analysis of 75 patients. *Klinische Padiatr.* 1996;208:236-241 (in German).
3. Millot F, Esperou H, Bordigoni P, et al. Allogeneic bone marrow transplantation for chronic myeloid leukemia in childhood: a report from the Societe Francaise de Greffe de Moelle et de Therapie Cellulaire (SFGM-TC). *Bone Marrow TransPlant.* 2003;32:993-999.
4. Cwynarski K, Roberts IA, Iacobelli S, et al. Stem cell transplantation for chronic myeloid leukemia in children. *Blood.* 2003;102: 1224-1231.
5. Gamis AS, Haake R, McGlave P, et al. Unrelated-donor bone marrow transplantation for Philadelphia chromosome-positive chronic myelogenous leukemia in children. *J Clin Oncol.* 1993; 11:834-838.
6. Baccarani M, Saglio G, Goldman J, et al. Evolving concepts in the management of chronic myeloid leukemia: recommendations from an expert panel on behalf of the European LeukemiaNet. *Blood.* 2006;108:1809-1820.
7. Druker BJ, Talpaz M, Resta DJ, et al. Efficacy and safety of a specific inhibitor of the BCR-ABL tyrosine kinase in chronic myeloid leukemia. *N Engl J Med.* 2001;344:1031-1037.
8. Gratwohl A, Brand R, Apperley J, et al. Allogeneic hematopoietic stem cell transplantation for chronic myeloid leukemia in Europe 2006: transplant activity, long-term data and current results. An analysis by the Chronic Leukemia Working Party of the European Group for Blood and Marrow Transplantation (EBMT). *Haematologica.* 2006;91:513-521.
9. Rousselot P, Huguet F, Rea D, et al. Imatinib mesylate discontinuation in patients with chronic myelogenous leukemia in complete molecular remission for more than 2 years. *Blood.* 2007;109:58-60.
10. Guastafierro S, Falcone U, Celentano M, et al. Is it possible to discontinue imatinib mesylate therapy in chronic myeloid leukemia patients with undetectable BCR/ABL? A case report and a review of the literature. *Leuk Res.* 2009;33:1079-1081.
11. Speck B, Bortin MM, Champlin R, et al. Allogeneic bone marrow transplantation for chronic myelogenous leukaemia. *Lancet.* 1984;1:665-668.
12. Glucksberg H, Storb R, Fefer A, et al. Clinical manifestations of graft-versus-host disease in human recipients of marrow from HLA-matched sibling donors. *TransPlantation.* 1974;18:295-304.
13. Storb R, Prentice RL, Thomas ED. Marrow transplantation for treatment of aplastic anemia: an analysis of factors associated with graft rejection. *N Engl J Med.* 1977;296:61-66.
14. Dominietto A, Lamparelli T, Raiola AM, et al. Transplant-related mortality and long-term graft function are significantly influenced by cell dose in patients undergoing allogeneic marrow transplantation. *Blood.* 2002;100:3930-3934.
15. Sierra J, Storer B, Hansen JA, et al. Transplantation of marrow cells from unrelated donors for treatment of high-risk acute leukemia: the effect of leukemic burden, donor HLA-matching, and marrow cell dose. *Blood.* 1997;89:4226-4235.
16. Gengozian N, Makinodan T. Mortality of mice as affected by variation of the x-ray dose and number of nucleated rat bone marrow cells injected. *Cancer Res.* 1957;17:970-975.
17. Uharek L, Gassmann W, Glass B, et al. Influence of cell dose and graft-versus-host reactivity on rejection rates after allogeneic bone marrow transplantation. *Blood.* 1992;79:1612-1621.
18. Pocock C, Szydlo R, Davis J, et al. Stem cell transplantation for chronic myeloid leukaemia: the role of infused marrow cell dose. *Hematol J.* 2001;2:265-272.
19. Maywald O, Pfirrmann M, Berger U, et al. Cytogenetic response to prior treatment with interferon-alpha is predictive for survival after allogeneic hematopoietic stem cell transplantation in chronic myeloid leukemia. *Leukemia.* 2006;20:477-484.
20. Weisser M, Schleuning M, Haferlach C, et al. Allogeneic stem cell transplantation provides excellent results in advanced stage

chronic myeloid leukemia with major cytogenetic response to pretransplant imatinib therapy. *Leuk LymPhoma*. 2007;48:295-301.
21. Lee SJ, Kukreja M, Wang T, et al. Impact of prior imatinib mesylate on the outcome of hematopoietic cell transplantation for chronic myeloid leukemia. *Blood*. 2008;112:3500-3507.
22. Champagne MA, Capdeville R, Krailo M, et al. Imatinib mesylate (STI571) for treatment of children with Philadelphia chromosome–positive leukemia: results from a Children's Oncology Group phase 1 study. *Blood*. 2004;104:2655-2660.
23. Millot F, Guilhot J, Nelken B, et al. Imatinib mesylate is effective in children with chronic myelogenous leukemia in late chronic and advanced phase and in relapse after stem cell transplantation. *Leukemia*. 2006;20:187-192.
24. Druker BJ, Guilhot F, O'Brien SG, et al. Five-year follow-up of patients receiving imatinib for chronic myeloid leukemia. *N Engl J Med*. 2006;355:2408-2417.
25. Larson RA, Druker BJ, Guilhot F, et al. Imatinib pharmacokinetics and its correlation with response and safety in chronic-phase chronic myeloid leukemia: a subanalysis of the IRIS study. *Blood*. 2008;111:4022-4028.
26. Picard S, Titier K, Etienne G, et al. Trough imatinib plasma levels are associated with both cytogenetic and molecular responses to standard-dose imatinib in chronic myeloid leukemia. *Blood*. 2007;109:3496-3499.
27. Kimoto T, Inoue M, Kawa K. Growth deceleration in a girl treated with imatinib. *Int J Hematol*. 2009;89:251-252.

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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, *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] 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.

References

1. Garrido F, Ruiz-Cabello F, Cabrera T, et al. Implications for immunosurveillance of altered HLA class I phenotypes in human tumours. *Immunol Today*. 1997;18(2):89-95.
2. Seliger B, Cabrera T, Garrido F, Ferrone S. HLA class I antigen abnormalities and immune escape by malignant cells. *Semin Cancer Biol*. 2002;12(1):3-13.
3. Marincola FM, Jaffee EM, Hicklin DJ, Ferrone S. Escape of human solid tumors from T-cell recognition: molecular mechanisms and functional significance. *Adv Immunol*. 2000;74:181-273.
4. Wetzler M, Baer MR, Stewart SJ, et al. HLA class I antigen cell surface expression is preserved on acute myeloid leukemia blasts at diagnosis and at relapse. *Leukemia*. 2001;15(1):128-133.
5. Masuda K, Hiraki A, Fujii N, et al. Loss or down-regulation of HLA class I expression at the allelic level in freshly isolated leukemic blasts. *Cancer Sci*. 2007;98(1):102-108.
6. Takahashi Y, McCoy JP, Jr., Carvallo C, et al. In vitro and in vivo evidence of PNH cell sensitivity to immune attack after nonmyeloablative allogeneic hematopoietic cell transplantation. *Blood*. 2004;103(4):1383-1390.
7. Sasazuki T, Juji T, Morishima Y, et al. Effect of matching of class I HLA alleles on clinical outcome after transplantation of hematopoietic stem cells from an unrelated donor. Japan Marrow Donor Program. *N Engl J Med*. 1998;339(17):1177-1185.
8. Nannya Y, Sanada M, Nakazaki K, et al. A robust algorithm for copy number

detection using high-density oligonucleotide single nucleotide polymorphism genotyping arrays. *Cancer Res*. 2005;65(14):6071-6079.

9. Torikai H, Akatsuka Y, Miyazaki M, et al. A novel HLA-A*3303-restricted minor histocompatibility antigen encoded by an unconventional open reading frame of human TMSB4Y gene. *J Immunol*. 2004;173(11):7046-7054.

10. Akatsuka Y, Nishida T, Kondo E, et al. Identification of a polymorphic gene, BCL2A1, encoding two novel hematopoietic lineage-specific minor histocompatibility antigens. *J Exp Med*. 2003;197(11):1489-1500.

11. Riddell SR, Greenberg PD. The use of anti-CD3 and anti-CD28 monoclonal antibodies to clone and expand human antigen-specific T cells. *J Immunol Methods*. 1990;128(2):189-201.

12. Kondo E, Topp MS, Kiem HP, et al. Efficient generation of antigen-specific cytotoxic T cells using retrovirally transduced CD40-activated B cells. *J Immunol*. 2002;169(4):2164-2171.

13. Takahashi Y, Harashima N, Kajigaya S, et al. Regression of human kidney cancer following allogeneic stem cell transplantation is associated with recognition of an HERV-E antigen by T cells. *J Clin Invest*. 2008;118(3):1099-1109.

14. Tuna M, Knuutila S, Mills GB. Uniparental disomy in cancer. *Trends Mol Med*. Feb 24 2009.

15. Koh LP, Rizzieri DA, Chao NJ. Allogeneic hematopoietic stem cell transplant using mismatched/haploidentical donors. *Biol Blood Marrow Transplant*. 2007;13(11):1249-1267.

16. Vago L, Perna SK, Zanussi M, et al. Loss of mismatched HLA in leukemia after stem-cell transplantation. *N Engl J Med*. 2009;361(5):478-488.

17. Khong HT, Restifo NP. Natural selection of tumor variants in the generation of "tumor escape" phenotypes. *Nat Immunol*. 2002;3(11):999-1005.

18. Ruggeri L, Capanni M, Urbani E, et al. Effectiveness of donor natural killer cell alloreactivity in mismatched hematopoietic transplants. *Science*. 2002;295(5562):2097-2100.

19. Velardi A, Ruggeri L, Mancusi A, et al. Clinical impact of natural killer cell reconstitution after allogeneic hematopoietic transplantation. *Semin*

Immunopathol. 2008;30(4):489-503.

20. Kolb HJ. Graft-versus-leukemia effects of transplantation and donor lymphocytes. *Blood*. 2008;112(12):4371-4383.

21. Huang XJ, Liu DH, Liu KY, Xu LP, Chen H, Han W. Donor lymphocyte infusion for the treatment of leukemia relapse after HLA-mismatched/haploidentical T-cell-replete hematopoietic stem cell transplantation. *Haematologica*. 2007;92(3):414-417.

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