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Figure legends

Figure 1. Semi-landmark plots illustrating the effects of acute GVHD on overall survival (A), disease-associated mortality (B), and treatment-related mortality (C).

Figure 2. Impact of the grade of acute GVHD on overall survival in each stratified category. Effects of grade 1–2 (Panel A) and grade 3–4 acute GVHD (Panel B) on overall survival are shown as forest plots. Square boxes on lines indicate hazard ratios compared with “no acute GVHD group”, and horizontal lines represent the corresponding 95% confidential intervals. Abbreviations used are the same as described in the footnotes to Tables 1 and 2.

Figure 3. Semi-landmark plots illustrating impact of chronic GVHD on overall survival (A), disease-associated mortality (B), and treatment-related mortality (C).

Table 1. Characteristics of patients and transplants

Variables	No. of patients (%) (n = 294)
Age group at transplant (years)	
<=30	7 (2)
>30-40	30 (10)
>40-50	109 (37)
>50-60	123 (42)
>60	25 (9)
Sex	
Male	158 (54)
Female	136 (46)
Disease status	
Complete remission	99 (34)
Not in complete remission	178 (61)
Unknown	17 (6)
Conditioning regimen	
Myeloablative	102 (34)
Reduced-intensity	128 (44)
Unclassifiable	64 (22)
GVHD prophylaxis	
Cyclosporine-based	195 (66)
Tacrolimus-based	94 (32)
Others	5 (2)
Source of stem cells	
Bone marrow	132 (45)
Peripheral blood	111 (38)
Bone marrow + peripheral blood	2 (1)
Cord blood	49 (17)
Type of donor*	
HLA-matched related	132 (45)
HLA-mismatched related	31 (11)
Unrelated, bone marrow	82 (28)
Unrelated, cord blood	49 (17)
Time from diagnosis to transplant	
<=6 months	141 (48)
>6 months	141 (48)
Uncertain/missing	12 (4)
Year of transplant	
1995-1999	22 (7)
2000-2002	91 (31)
2003-2005	181 (62)
Follow-up of survivors	
Median time† (range)	42.8 (1.5–102.3)

Data are numbers (%) unless specified otherwise.

Abbreviations: Cyclosporine-based, cyclosporine with or without other agents; tacrolimus-based, tacrolimus with or without other agents.

*HLA compatibility was defined according to the results of serologic or low-resolution molecular typing for HLA-A, B and DR antigens. †Data are expressed in months.

Table 2. Effect of acute GVHD on overall survival, disease-associated mortality, and treatment-related mortality after allogeneic hematopoietic cell transplantation for adult T-cell leukemia

Outcome	Univariable analysis		Multivariable analysis	
	Hazard ratio (95% CI)	P	Hazard ratio (95% CI)	P
Overall survival*				
Grade 1–2 acute GVHD vs no acute GVHD	0.60 (0.42-0.85)	0.004	0.65 (0.45-0.93)	0.018
Grade 3–4 acute GVHD vs no acute GVHD	1.38 (0.94-2.01)	0.099	1.64 (1.10-2.42)	0.014
Disease-associated mortality†				
Grade 1–2 acute GVHD vs no acute GVHD	0.47 (0.28-0.79)	0.005	0.54 (0.32-0.92)	0.023
Grade 3–4 acute GVHD vs no acute GVHD	0.41 (0.21-0.81)	0.010	0.44 (0.22-0.90)	0.024
Treatment-related mortality‡				
Grade 1–2 acute GVHD vs no acute GVHD	1.13 (0.67-1.89)	0.649	1.22 (0.72-2.07)	0.461
Grade 3–4 acute GVHD vs no acute GVHD	3.34 (1.94-5.74)	<0.001	3.50 (2.01-6.11)	<0.001

Abbreviations: GVHD, graft-versus-host disease; CI, confidence interval.

*Other significant variables were; sex of recipient, female (reference, 1.00), male (HR, 1.70; 95% CI, 1.24–2.32; p 0.001); achievement of complete remission, complete remission (reference, 1.00), status other than complete remission (HR, 2.05; 95% CI, 1.44–2.92; p 0.001), status not known, (HR, 2.21; 95% CI, 1.15–4.22; p 0.017); type of donor, HLA-matched related donor (reference, 1.00), HLA-mismatched related donor (HR, 1.71; 95% CI, 1.04–2.84; p 0.036), unrelated donor of bone marrow (HR, 1.39; 95% CI, 0.94–2.06; p 0.096), unrelated cord blood (HR, 1.86; 95% CI, 1.22–2.83; p 0.004).

†Other significant variables were; achievement of complete remission, complete remission (reference, 1.00), status other than complete remission (HR, 2.98; 95% CI, 1.62–5.47; p 0.001), status not known, (HR, 0.96; 95% CI, 0.21–4.49; p 0.963); type of donor, HLA-matched related donor (reference, 1.00), HLA-mismatched related donor (HR, 2.14; 95% CI, 1.00–4.55; p 0.049), unrelated donor of bone marrow (HR, 1.45; 95% CI, 0.81–2.61; p 0.214), unrelated cord blood (HR, 1.25; 95% CI, 0.63–2.49; p 0.517).

‡Another significant variable was; achievement of complete remission, complete remission (reference, 1.00), status other than complete remission (HR, 1.17; 95% CI, 0.74–1.84; p 0.498), status not known, (HR, 2.31; 95% CI, 1.04–5.15; p 0.040).

Table 3. Effect of chronic GVHD on overall survival, disease-associated mortality, and treatment-related mortality after allogeneic hematopoietic cell transplantation for adult T-cell leukemia

Outcome	Univariable analysis		Multivariable analysis	
	Hazard ratio (95% CI)	P	Hazard ratio (95% CI)	P
Overall survival*				
Limited chronic GVHD vs no chronic GVHD	0.71 (0.34-1.47)	0.353	0.72 (0.35-1.50)	0.385
Extensive chronic GVHD vs no chronic GVHD	1.45 (0.90-2.35)	0.131	1.40 (0.86-2.30)	0.176
Disease-associated mortality†				
Limited chronic GVHD vs no chronic GVHD	0.45 (0.14-1.46)	0.183	0.45 (0.14-1.44)	0.178
Extensive chronic GVHD vs no chronic GVHD	0.81 (0.39-1.67)	0.563	0.80 (0.39-1.64)	0.536
Treatment-related mortality¶				
Limited chronic GVHD vs no chronic GVHD	1.59 (0.64-3.95)	0.316	1.56 (0.63-3.87)	0.342
Extensive chronic GVHD vs no chronic GVHD	2.85 (1.41-5.77)	0.004	2.75 (1.34-5.63)	0.006

Abbreviations: GVHD, graft-versus-host disease; CI, confidence interval.

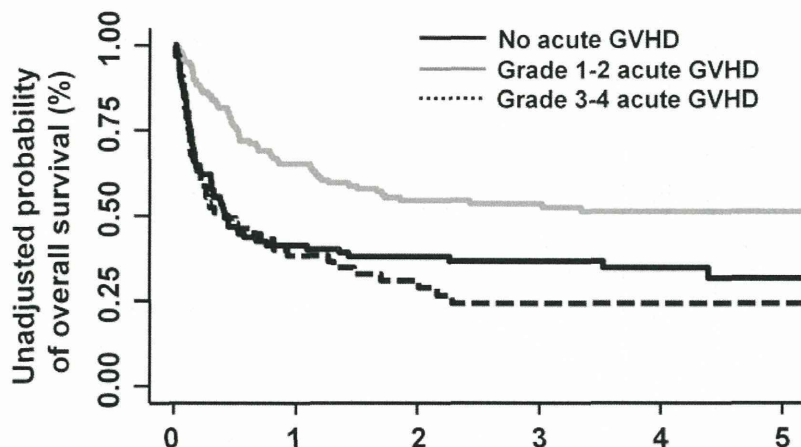
*There was no significant variable.

†There was no significant variable.

¶There was no other significant variable.

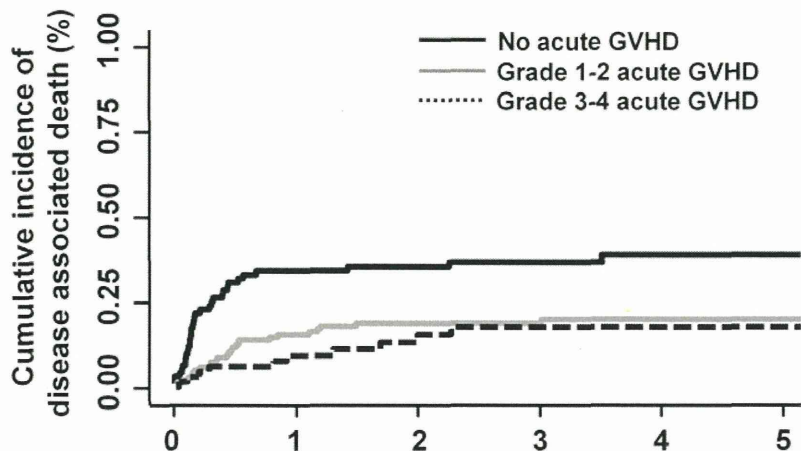
Figure 1.

A



	Years from onset/landmark					
Number at risk	0	1	2	3	4	5
No acute GVHD	92	37	32	23	12	7
Grade 1-2 aGVHD	137	83	65	48	29	19
Grade 3-4 aGVHD	65	23	14	8	4	3

B



	Years from onset/landmark					
Number at risk	0	1	2	3	4	5
No acute GVHD	88	36	32	23	12	7
Grade 1-2 aGVHD	134	80	65	48	29	19
Grade 3-4 aGVHD	64	23	14	8	4	3

Figure 1. (continued)

C

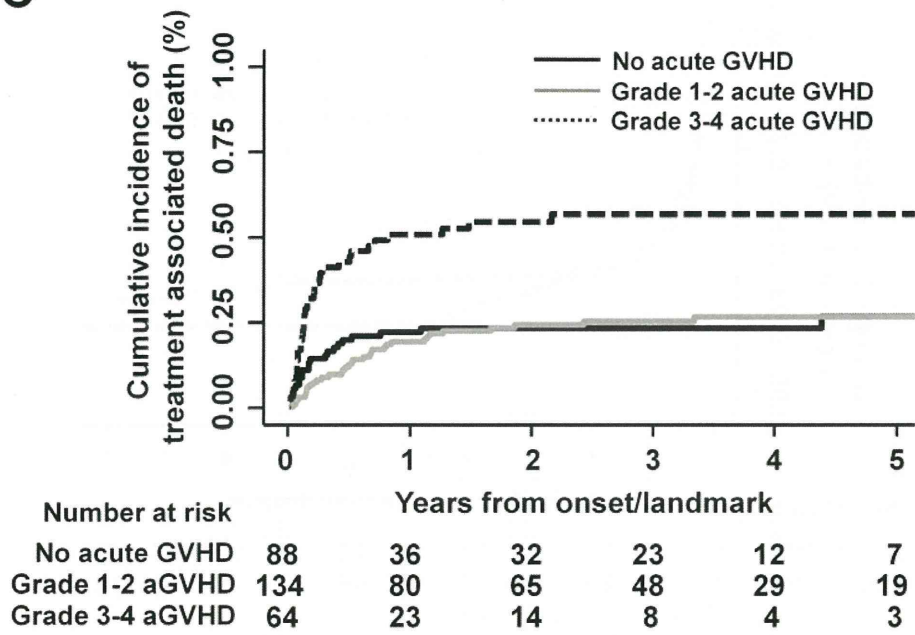


Figure 2.

Panel A.

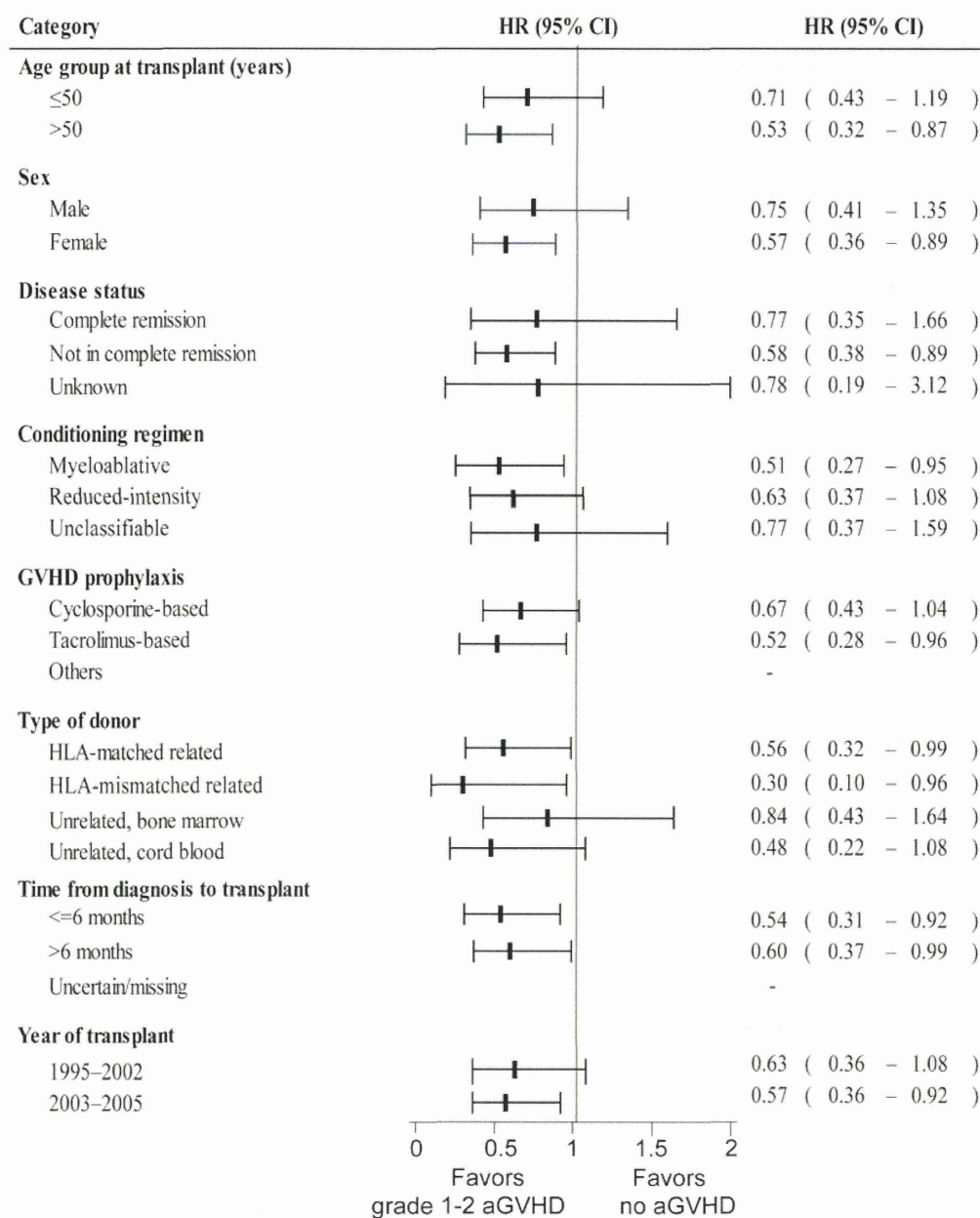


Figure 2. (continued)

Panel B.

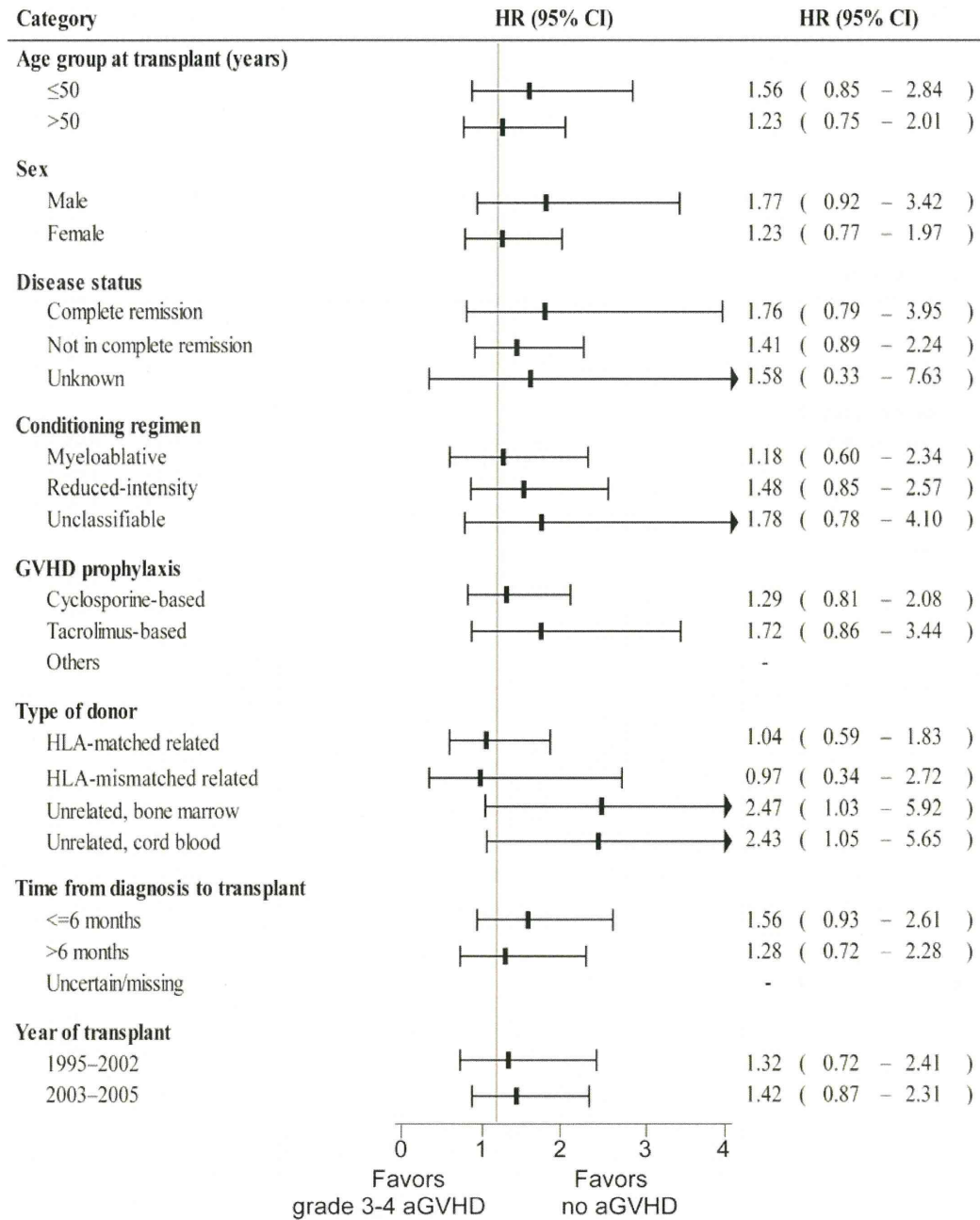
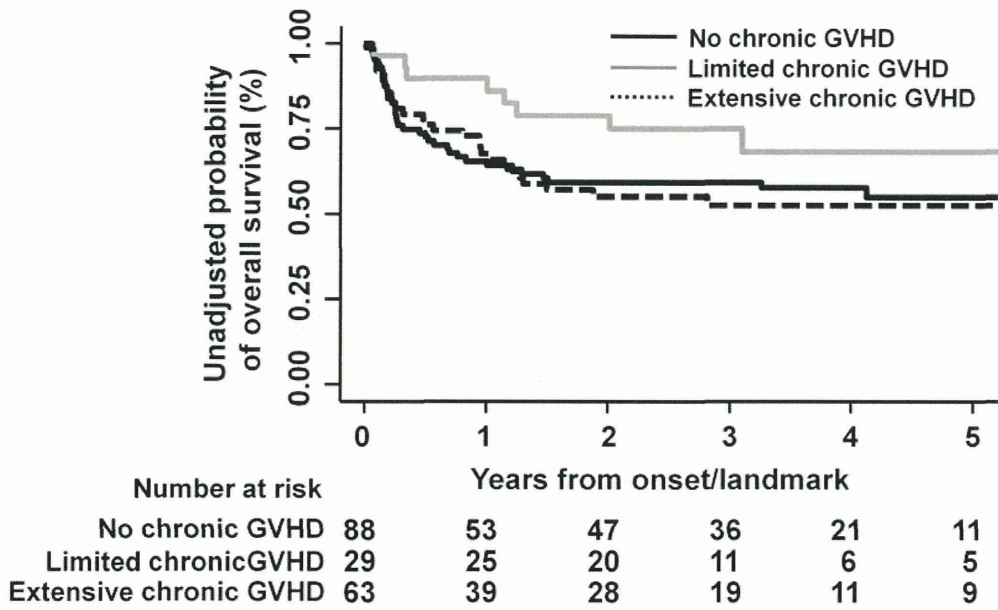


Figure 3.

A



B

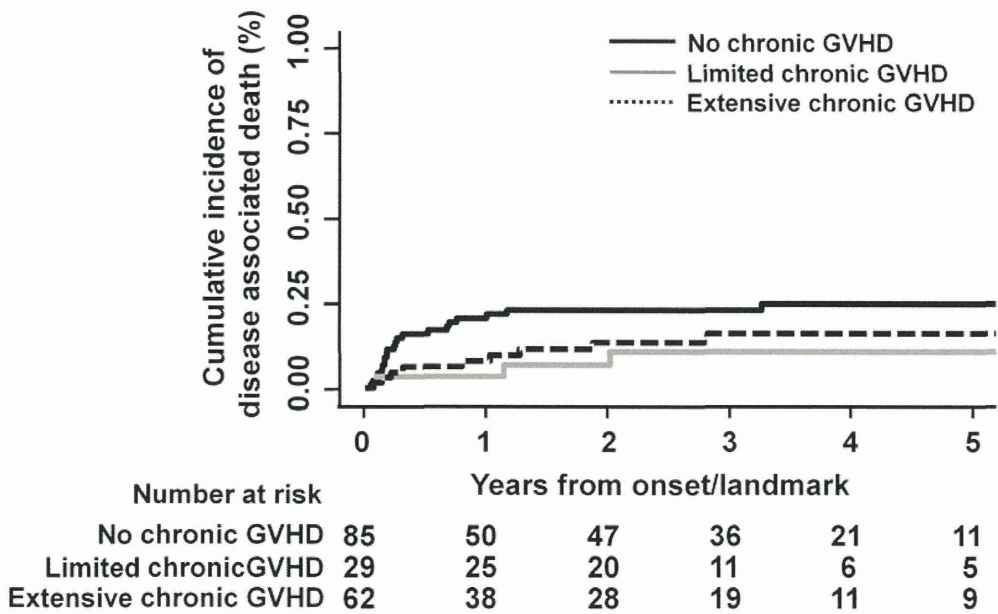
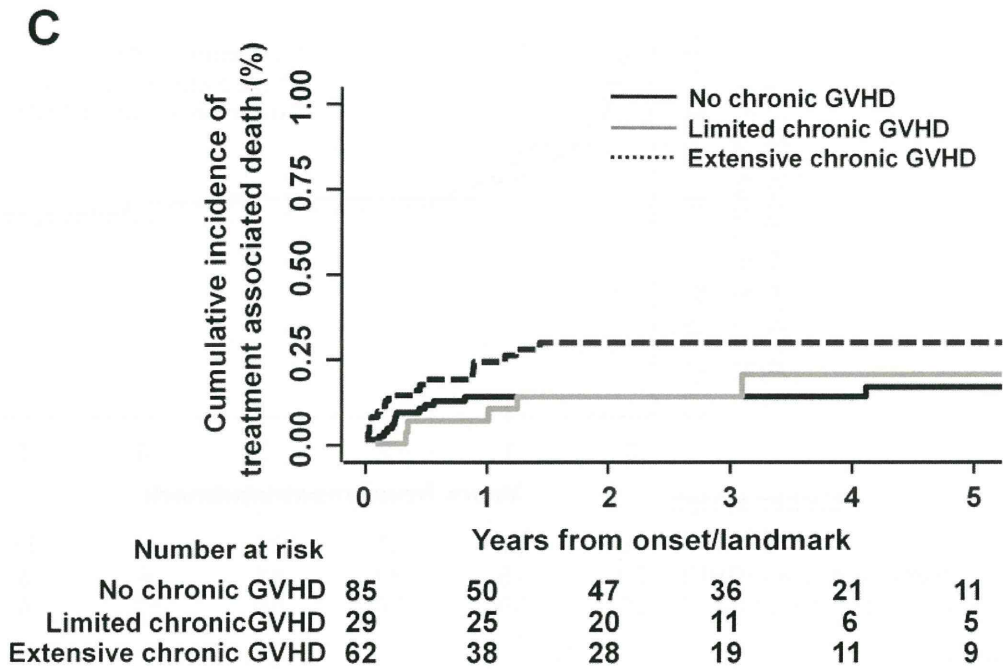


Figure 3. (continued)



Allogeneic stem cell transplantation as treatment for heavily treated, refractory acute graft-versus-host disease after HLA-mismatched stem cell transplantation

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Objective. No effective treatment has been established for patients with steroid-refractory acute graft-versus-host disease (GVHD). Recently, we demonstrated in a murine tandem bone marrow transplantation model that life-threatening GVHD established by the first bone marrow transplantation was successfully treated by engraftment of a second donor graft after reduced-intensity conditioning. We named the effect by which allografts counteract GVHD “graft-versus-GVHD.”

Materials and Methods. To investigate the efficacy of graft-versus-GVHD treatment clinically, 16 patients who developed, after human leukocyte antigen–mismatched stem cell transplantation, severe GVHD, refractory to three to five lines of GVHD-specific treatments, underwent 17 allogeneic stem cell transplantations using reduced-intensity conditioning regimens with grafts from a second donor.

Results. Among the 15 transplantations that could be evaluated, rescue donor grafts were engrafted in 11 cases and rejected in 4 cases. For patients who achieved rescue donor engraftment, the response rate was 90.9% (eight complete response, two partial response, and one stable disease). Six of the eight patients with complete response survived without GVHD symptoms, with a median follow-up of 2128 days. No new development of GVHD by the second graft was observed. No patients had recurrence of the original malignant disease. In contrast, no long-term survivors were observed in patients who rejected rescue donor grafts.

Conclusions. We propose here a novel graft-versus-GVHD treatment to treat refractory GVHD, and these results strongly suggest that GVHD can be successfully treated by eliminating the harmful lymphocytes responsible for GVHD by a second allogeneic stem cell transplantation. © 2011 ISEH - Society for Hematology and Stem Cells. Published by Elsevier Inc.

Graft-versus-host-disease (GVHD) is a major obstacle to successful allogeneic bone marrow transplantation (BMT), and greatly limits the applications and efficacy of allogeneic BMT. In particular, for steroid-refractory GVHD, no consensus treatment has been established [1,2], although a number of therapeutic approaches, including mesenchymal stem cells, pentostatin, infliximab, and a variety of monoclonal antibodies, have been reported [3–7].

We and others have attempted to treat patients with severe GVHD by second transplantation using autologous or syngeneic hematopoietic cells to ablate the lymphoid cells responsible for GVHD [8–10]. Although severe GVHD resolved or partially improved after these transplantations, relapse of the original tumor occurred in the majority of patients.

Therefore, we intended to use a second allogeneic donor as a graft source for rescue transplantation against GVHD. We recently demonstrated in a murine tandem BMT model where the three mouse strains shared one major histocompatibility complex haplotype and the other major histocompatibility complex haplotype was different, that

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life-threatening GVHD established by the first BMT using myeloablative conditioning was successfully treated by engraftment of a second donor graft using reduced-intensity conditioning treatment [11]. In allogeneic stem cell transplantation (SCT) for autoimmune diseases, donor lymphocytes are considered to have the capacity to eliminate all residual self-reactive host lymphocytes through a process known as graft-versus-autoimmunity effects [12], with analogy to graft-versus-leukemia (GVL) in leukemia. Thus, we named the effects by which second allografts counteract GVHD through permanent elimination or transient reduction of first donor harmful lymphocytes, “graft-versus-GVHD” [11].

In addition, clinically, we recently developed a novel unmanipulated human leukocyte antigen (HLA)-haploidentical nonmyeloablative SCT using a conditioning treatment consisting of fludarabine + busulfan + anti-T-lymphocyte globulin (ATG), and GVHD prophylaxis consisting of tacrolimus (FK506) + methylprednisolone (mPSL) (1 mg/kg), in which the incidence of acute GVHD was only 20% [13]. As some GVHDs occurred after donor lymphocyte infusion or rapid tapering of immunosuppressive agents for early relapse or severe viral infections, the actual incidence of GVHD was estimated to be 10%; therefore, we applied this HLA-haploidentical nonmyeloablative SCT to rescue transplantation for refractory GVHD.

In the present study, we investigated whether second allogeneic SCT could treat patients with severe, steroid-refractory GVHD.

Materials and methods

Patients

From February 2001 to December 2008, 320 patients underwent allogeneic SCT at Osaka University Hospital or at the Hospital of Hyogo College of Medicine. Among them, 16 consecutive adult patients who developed severe refractory GVHD after HLA-mismatched SCT underwent a second allogeneic SCT to treat GVHD. All of these patients were in remission at the time of rescue transplantation. The major objectives in this study were improved GVHD and survival at 6 months. GVHD was diagnosed from a biopsy of at least one involved organ. Patients with severe GVHD (\geq grade II) who did not respond to mPSL (\geq 2 mg/kg) or who had recurrent GVHD at a dose of steroids \geq 1 mg/kg mPSL were eligible for the study; however, patients who were finally enrolled received a median of four (range of two to five) lines of GVHD-specific treatments, including tumor necrosis factor blocker, ATG, and mycophenolate mofetil, by the time of the rescue transplantation (Table 1). In general, GVHD occurring after HLA-mismatched SCT progresses very rapidly, and quickly becomes irreversible; therefore, in the first SCT inducing GVHD, when the manifestations of GVHD worsened during 3 days of treatment, other immunosuppressive agents were added [14], sometimes in combination. Regarding the eligibility criteria for the rescue transplantation, patients who had HLA-identical or HLA 1–3 antigen-mismatched related donors were eligible.

Patients were not eligible for rescue transplantation if they had severe renal, heart, or lung disease: serum creatinine level $>$ 1.5 times the normal upper limit, ejection fraction $<$ 50% on an echocardiogram, or oxygen saturation $<$ 93%, respectively. Patients were not eligible for rescue transplantation if they had severe liver disease that was considered to be caused by diseases other than GVHD; total bilirubin level $>$ 2.0 mg/dL, and aspartate aminotransferase $>$ 2.5 times the normal upper limit.

The characteristics of the patients and first transplantation inducing severe GVHD are shown in Table 1. Because one patient underwent allogeneic rescue SCT twice, 17 graft-versus-GVHD treatments were performed. Among the 16 patients, 14 had developed acute GVHD after allogeneic SCT, including 3 patients who had developed recurrent acute GVHD $>$ 100 days after transplantation and 2 after donor lymphocyte infusion. Institutional review board approval was obtained for the treatment protocol, and written informed consent was obtained from the patients and their families.

Four patients underwent the first transplantation (inducing severe GVHD) using a graft from an HLA 2–3 antigen-mismatched donor, and underwent the second (rescue) transplantation using a graft from an HLA-matched or 1 antigen-mismatched donor (Table 2). The donor in the first transplantation was selected for the following reasons. We recently reported that unmanipulated HLA-haploidentical SCT was useful for treating patients with hematologic malignant diseases in the advanced stage [13,15,16]. Thus, in our HLA-haploidentical SCT protocol, patients with a full-blown relapse can undergo allogeneic SCT using a graft from an HLA-haploidentical donor, even when an HLA-matched (or 1 antigen-mismatched) related donor is available. Such decisions were made at the recommendation of the physicians and with the concurrence of the patient and family members after considering the overall risks of recurrent malignancy, graft rejection, and severe GVHD with the two different types of donors.

Rescue transplantation procedure

Details of the rescue transplantation are shown in Table 2. Median interval between the previous allogeneic SCT and the rescue transplantation was 59 days (range, 32–481 days). All patients received a reduced-intensity conditioning treatment. The conditioning consisted of 30 mg/m² fludarabine intravenously for 3 consecutive days on days –6 to –4, ATG (Fresenius) 2 mg/kg/day for 4 days (day –4 to day –1) with or without total body irradiation 3 Gy on day 0. Eight patients could not receive total body irradiation because they had received total body or local irradiation as previous treatments. One patient (no. 10–2) who rejected the first rescue transplantation received thiotepea 10 mg/kg on day –2 and total body irradiation 4 Gy on day –1 in addition to fludarabine and ATG. In all cases, peripheral blood stem cells were used as the stem cell source.

GVHD prophylaxis was performed with FK506 and mPSL (1 mg/kg), as reported previously [13]. In brief, FK506 treatment was initiated the day before transplantation and given at a dose of 0.02 mg/kg/day as a continuous infusion. The target blood concentration of FK506 was set between 8 and 10 ng/mL until day 30, and was thereafter tapered in the absence of acute GVHD. Patients received intravenous FK506 therapy until they could reliably receive oral medications after transplantation. Intravenous administration of mPSL was started at a dose of 1 mg/kg/day from day –4. mPSL tapering was started in the third week and was performed relatively rapidly until day 30 using the serum soluble

Table 1. Patients' characteristics and first transplantation inducing severe GVHD

No	Sex/Age	Disease	Disease status	Conditioning regimen	Donor	HLA disparity	PS	grade	Stage			prior treatment for GVHD
									skin	gut	liver	
1	23/F	ALL	PR	full	Mother	2/2 [†]	50	II	3	1	0	MTX, MMF, mPSL(2), Flu,
2	17/M	LBL	Re3	full	Cousin	2/3	10	III	3	3	1	Flu, ATG, MTX, MMF(inc)
3	33/M	ALL	PR	full	Sibling	3/3	20	III	3	4	0	MTX, MMF(inc), Flu, ATG,
4	37/M	MDS	RAEB	full	Offspring	3/3	20	III	3	4	0	Flu, MMF(inc), infliximab, ATG, pulse mPSL
5	25/M	CML	Re(autoBM)*	full	Sibling	2/2	70	II	3	0	0	PSL(inc), MMF
6	21/F	NHL	CR2(autoPB)	full	Mother	2/0	50	II	3	0	0	MMF, infliximab
7	19/M	HD	RR	full	Father	3/2	50	IV	4	0	0	MTX, ATG, infliximab
8	22/M	ALL	Re2	full	Sibling	3/2	10	III	0	3	3	infliximab, ATG, pulse mPSL, MTX, basiliximab
9	19/F	CML	BC	full	Sibling	2/2	70	II	3	0	0	infliximab, ATG, pulse mPSL, MTX, MMF(inc)
10-1	19/M	SNCL	IF	full	Sibling	2/2	50	III	3	4	0	MTX, infliximab, pulse mPSL, ATG
10-2	19/M	SNCL	IF	RIST	Mother	2/2	30	III	3	2	0	mPSL(inc), infliximab, MMF, ATG
11	41/F	LAHS	IF	full	Offspring	2/3	20	III	3	2	3	ATG, infliximab, MMF, pulse mPSL
12	21/F	AML	Re(alloBM)	RIST	Father	3/3	20	III	2	2	2	infliximab, pulse mPSL, MMF, ATG
13	49/M	CML	CP	RIST	Offspring	2/3	30	IV	4	2	0	ATG, MTX, infliximab, pulse mPSL
14	19/F	ALL	Re2	full	Sibling	3/1	40	III	2	3	1	pulse mPSL, MMF, etanercept, ATG
15	47/F	ALL	Re(alloPB)	RIST	UCB	4/2	40	III	3	3	0	etanercept, MMF, pulse mPSL
16	31/F	ALL	RR	full	Sibling	3/2	60	III	2	2	0	PSL(inc), pulse mPSL, MTX

AML, acute myeloid leukemia; ALL, acute lymphoid leukemia; CML, chronic myeloid leukemia; MDS, myelodysplastic syndrome; NHL, non-Hodgkin's lymphoma; HD, Hodgkin's lymphoma; SNCL, small non-cleaved lymphoma; LAHS, lymphoma-associated hemophagocytic syndrome; CR2, second complete remission; PR, partial remission; Re, relapse; Re2 or Re3, second or third relapse; RR, resistant relapse; RAEB, refractory anemia with excess of blasts; CP, chronic phase; BC, blastic crisis; IF, induction failure; full, full regimen; RIST, reduced intensity of conditioning treatment; PS, Karnofsky performance status; MTX, methotrexate; MMF, mycophelate mofetil; mPSL(2), methylprednisolone 2 mg/kg; pulse mPSL, pulse therapy of methylprednisolone; Flu, fludarabine; ATG, anti-T-lymphocyte globulin; inc, increase in dose; autoBM, autologous bone marrow transplantation; autoPB, autologous peripheral blood stem cell transplantation; alloBM, allogeneic bone marrow transplantation; alloPB, allogeneic peripheral blood stem cell transplantation.

*Transplantation in parentheses indicates previous stem cell transplantation.

[†]Numbers before or after a slash indicate mismatched HLA antigens in GVH or HVG directions, respectively.

Table 2. Details of the rescue transplantation

No	Interval between 2 transplantations (days)	Donor			Conditioning treatment	Cell dose		Engraftment of rescue graft	days	Hematological recovery		GvGVHD effect	survival (days)	Cause of death
		relationship	Sex/ Age	HLA disparity		CD34 cells × 10 ⁶ /kg	CD3 cells × 10 ⁸ /kg			Neu > 0.5 × 10 ⁹ /l (days)	PLT > 20 × 10 ⁹ /l (days)			
1	94	Sibling	F/25	2/2	chemo	3.90	1.61	+	15	9	101	complete	+3304	-
2	40	Sibling	F/22	0/0	chemo	6.60	5.81	NE		8	-	NA	10	TMA
3	145	Mother	F/58	1/1	chemo	3.76	5.38	NE		-	-	NA	13	Renal failure
4	40	Offspring	M/12	3/3	chemo	16.50	4.19	+	20	10	-	partial	135	GVHD
5	481	Mother	F/55	2/2	chemo+TBI	3.90	2.50	+	34	not decreased	not decreased	complete	+2714	-
6	213	Sibling	F/23	1/0	chemo+TBI	5.20	6.71	+	29	10	36	complete	831	Cardiac failure
7	47	Mother	F/45	2/2	chemo	3.60	3.57	-		26	-	partial	76	Pneumonia
8	98	Mother	F/47	3/3	chemo+TBI	6.20	3.11	+	11	10	-	partial	23	Pneumonia
9	227	Mother	F/48	2/3	chemo+TBI	4.51	2.06	+	17	not decreased	not decreased	complete	+2170	-
10-1	59	Mother	F/51	2/2	chemo+TBI	2.80	2.12	-		not decreased	-	transient	+42	-
10-2	101	Mother	F/51	2/2	chemo+TBI*	2.30	2.27	+	14	9	32	complete	+2086	-
11	63	Sibling	M/37	0/0	chemo	7.10	1.71	-		not decreased	not decreased	partial	33	VOD
12	32	Mother	F/ 51	3/0	chemo+TBI	23.00	3.49	-		not decreased	-	partial	46	GVHD
13	36	Offspring	M/22	2/3	chemo+TBI	7.16	3.22	+	52	8	9	complete	+1637	-
14	59	Sibling	F/12	3/1	chemo+TBI	18.60	8.10	+	14	10	-	transient	72	TTP
15	49	Offspring	M/16	3/3	chemo	17.10	4.30	+	8	8	-	complete	163	Hepatic failure
16	39	Sibling	F/27	3/2	chemo	14.00	2.66	+	107	not decreased	16	complete	+490	-

chemo, chemotherapy consisting of fludarabine 30 mg/m² and anti-T-lymphocyte globulin; TBI, total body irradiation 3Gy; NE, not evaluable; not decreased, neutrophils or platelet counts did not decrease below 0.5 × 10⁹/l or 20 × 10⁹/l, respectively; GvGVHD effect, graft-versus-GVHD effect; complete, complete response; partial, partial response; TMA, thrombotic microangiopathy; VOD, hepatic veno-occlusive disease; TTP, thrombotic thrombocytopenic purpura.

*Thiotepa 10 mg/kg and TBI 4 Gy were given in addition to fludarabine and ATG.

interleukin-2 receptor level [17,18], as an indicator, and was thereafter continued carefully.

Acute GVHD was graded according to standard criteria [19] and GVHD beyond 100 days after transplantation was diagnosed based on the proposed National Institutes of Health criteria [20]. Patient status before rescue transplantation was assessed by the Karnofsky performance rating. We defined the response to treatment as follows: complete response: loss of all symptoms of acute GVHD; partial response: improvement of at least one GVHD grade; stable disease: no change in GVHD grade; progressive disease: worsening of GVHD. Regarding the assessment of GVHD after the rescue transplantation, if the symptoms of patients were considered to have been caused mainly by a complication other than GVHD, their GVHD stages were downgraded by one stage, according to the recommendation in the 1994 consensus conference on acute GVHD grading [21]. A diagnosis based on autopsy directly reflected the assessment of response.

Each patient was isolated in a laminar air-flow room and standard decontamination procedures were followed. Oral antibiotics (ciprofloxacin, vancomycin, amphotericin B) were administered to sterilize the bowel. Patients with negative cytomegalovirus (CMV) IgG titers received blood products from CMV seronegative donors. Intravenous immunoglobulin was administered at a minimum dose of 100 mg/kg every 2 weeks until day 100. Cotrimoxazole was given for at least 1 year for prophylaxis of *Pneumocystis jirovecii* infections. Acyclovir was administered at a dose of 1000 mg/day for 5 weeks after transplantation to prevent herpes simplex infections.

Ganciclovir 7.5 mg/kg divided in three doses per day was administered from day -10 to day -3 as prophylaxis for CMV infection. Thrombotic microangiopathy was diagnosed according to Zeigler's criteria [22], and based on the recommendations reported by Nishida et al. [23].

Chimerism analysis

Chimerism between the donor and recipient was analyzed as described previously [13]. Chimerism analysis was continued twice a week after transplantation until donor engraftment or rejection. Blood samples were analyzed to determine the degree of donor/recipient chimerism in the T-cell or neutrophil-enriched cell fraction, using polymerase chain reaction amplification of informative microsatellite regions, which identified differences between the donor and recipient (based on polymorphisms found in pretransplantation donor/recipient samples) [24]. To remove monocytes, KAC-2 silica beads (Japan Immunoresearch Laboratories Co., Ltd., Gunma, Japan) were mixed with heparinized peripheral blood and incubated at 37°C for 1 hour. To enrich T cells, a negative selection system (RosetteSep; StemCell Technologies) was used [25]. To obtain a T-cell-enriched cell fraction, a cocktail containing anti-CD16, anti-CD19, anti-CD36, and anti-CD56 antibodies was added to the blood samples after they were treated with Silica beads. After Ficoll-Paque (GE Healthcare, Little Chalfont, Buckinghamshire, UK) density gradient centrifugation, CD3⁺ cells were recovered from the Ficoll: plasma interface with a purity >95%. Neutrophils were recovered from the Ficoll:RBC interface with a purity >99%.

Statistical analysis

The protocol was designed as a phase II study with sufficient power to detect a response rate of $\geq 20\%$ with a standard error of 10%. Comparison of patients who did or did not achieve rescue

donor engraftment for the response for GVHD was evaluated using the χ^2 test. Survival data from patients achieving rescue donor engraftment or not were compared based on the results of log-rank tests. Results were considered significant at $p < 0.05$.

Data were "locked" for analysis on May 31, 2010.

Results

Engraftment of rescue donor grafts

To treat GVHD, patients received peripheral blood stem cells from a second allogeneic donor with a median of 6.40×10^6 (range, $2.30\text{--}23.00 \times 10^6$) CD34⁺ cells/kg, including a median of 3.22×10^8 (range, $1.61\text{--}8.10 \times 10^8$) CD3⁺ cells/kg, without T-cell depletion. As shown in Table 1, 16 patients received 17 rescue transplantations to treat GVHD. Because of a poor performance status at transplantation, two patients (nos. 2 and 3) died early (days 10 and 13, respectively) and could not be evaluated for the effects of rescue transplantation; therefore, data from 15 transplantations were analyzed.

Among the 15 transplantations that could be evaluated, rescue donor grafts engrafted in 11 cases, but not in 4 cases. T-cell engraftment preceded neutrophil engraftment (data not shown). In chimerism analysis, all patients showed 100% first donor chimerism in both T-cell and myeloid cell components before the rescue transplantation. It was difficult to obtain continuous chimerism data between first and second (rescue) donors within 1 week after transplantation because of lymphocytopenia. Changes of T-cell chimerism of patients, in whom the chimeric status could be consecutively measured, are shown in Figure 1. In the four patients rejecting a rescue graft, although transiently increasing up to 35% on day 4, rescue donor-derived T cells, thereafter decreased and became undetectable up to 2 weeks after transplantation. Regarding patients who achieved engraftment, donor T-cell chimerism rapidly or gradually increased after transplantation, and full T-cell chimerism of the rescue donor was achieved in a median of 15 days (range, 7–106 days).

Regarding neutrophil recovery, in 6 of the 15 patients, absolute neutrophil counts did not decrease to $< 0.5 \times 10^9/L$, and in the remaining 9 patients, absolute neutrophil counts increased to $> 0.5 \times 10^9/L$ at a median of 10 days (range, 8–26 days). The platelet counts did not decrease to $< 20 \times 10^9/L$ in three patients (nos. 5, 10–2, and 12). Among the remaining 12 patients, platelet recovery occurred in 5 patients at a median of 32 days (range, 9–101 days), but not in the remaining 7 patients because of early death or subsequent transplantation.

Graft-versus-GVHD effects

Clinical effects of rescue transplantation are shown in Table 3. For successful graft-versus-GVHD treatment, engraftment of the rescue donor graft was mandatory in our murine model [11], in which immunosuppressive agents were not used. In the present clinical study, in which immunosuppressive agents

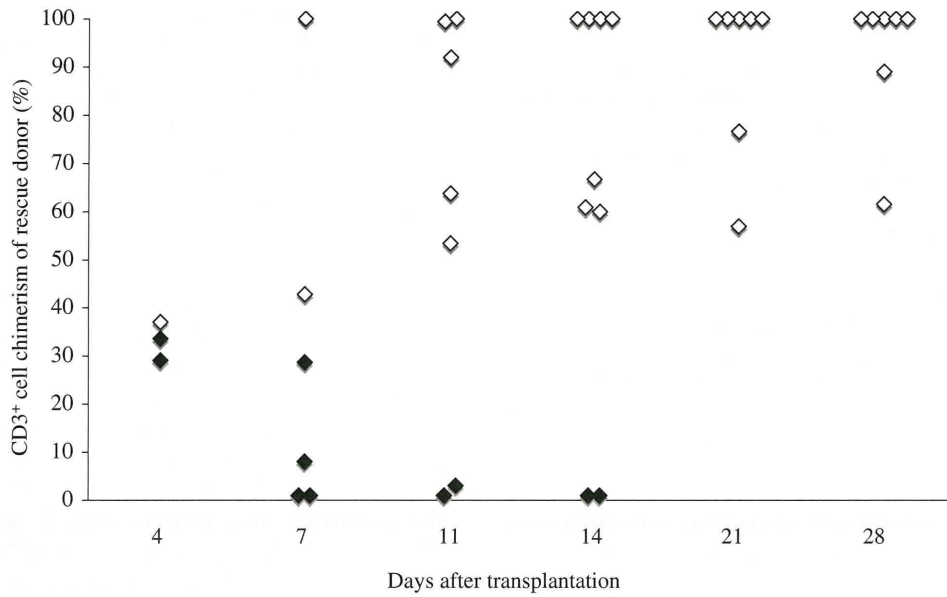


Figure 1. T-cell chimerism between first and second (rescue) donors in patients who did or did not achieve rescue donor engraftment. Open or closed diamonds denote patients who did or did not achieve rescue donor engraftment, respectively.

were naturally used in the transplantation, the response rate for patients achieving rescue donor engraftment or not was 90.9% (eight complete response, two partial response, and one stable disease) and 50% (one complete response, one partial response and one stable disease), respectively. Patients achieving rescue donor engraftment tended to show a higher response than patients not achieving engraftment ($p = 0.080$, χ^2 test). For the response of each organ, patients achieving rescue donor engraftment showed a significantly higher response with cutaneous GVHD than patients not achieving engraftment

($p = 0.016$), but there was no significant difference in response for intestinal and hepatic GVHDs between patients who did and did not achieve rescue donor engraftment. Regardless of achieving engraftment of the rescue donor graft, most GVHD symptoms began to improve during the conditioning treatment, and continued to improve by 1 week after transplantation. Thereafter, in patients who achieved rescue donor engraftment, the majority of GVHD symptoms continued to improve and disappeared within 40 days after transplantation, whereas in patients not achieving engraftment, some GVHD

Table 3. Change of the severity of GVHD

No.	engraftment	stage			grade
		skin	gut	liver†	
1	yes	3 → 0 (19)*	1 → 0 (0)	0 → 0	II → 0 (19)
4	yes	3 → 0 (6)	4 → 2 (12)	0 → 0	III → III
5	yes	3 → 0 (19)	0 → 0	0 → 0	II → 0 (19)
6	yes	3 → 0 (21)	0 → 0	0 → 0	II → 0 (21)
8	yes	0 → 0	3 → 1 (9)	3 → 0 (11)	III → II (11)
9	yes	3 → 0 (4)	0 → 0	0 → 0	II → 0 (4)
10-2	yes	3 → 0 (7)	2 → 0 (15)	0 → 0	III → 0 (15)
13	yes	4 → 0 (38)	2 → 0 (20)	0 → 0	IV → 0 (38)
14	yes	2 → 0 (-5)	3 → 0 (30)	1 → 1	III → II (30)
15	yes	3 → 0 (10)	3 → 0 (30)	0 → 0	III → 0 (30)
16	yes	2 → 0 (-6)	2 → 0 (5)	0 → 0	III → 0 (5)
7	no	4 → 1 (2)	0 → 0	0 → 0	IV → I (2)
10-1	no	3 → 1 (5)	4 → 1 (5) → 2 (19)	0 → 0	III → II (5) → III(19)
11	no	3 → 0 (-5)	2 → 0 (-5)	3 → 0†	III → 0†
12	no	2 → 0 (10)	2 → 0 (15)	2 → 3 (4) → 2(13)	III → III

*Numbers in parentheses denote the day after rescue transplantation when the stage or grade of GVHD was changed.

†Staging of hepatic GVHD was decided based on the serum bilirubin levels. Patient No.11 had an increased bilirubin level and died on day 33, but the main cause of death of the patient was diagnosed from autopsied samples with hepatic veno-occlusive disease without no evidence of GVHD.

symptoms disappeared and others became stable or rebounded. Once a complete response was achieved, no rebound of GVHD occurred. In 8 patients who achieved rescue donor engraftment and who had a complete response, the median time for achieving a complete response was 19 days (range, 4–38 days) after transplantation. Among three patients not achieving a complete response despite rescue donor engraftment, one patient (no. 4) showed a complete response for cutaneous GVHD, but had continued diarrhea. The diarrhea was diagnosed to be mainly caused by thrombotic microangiopathy because of partial improvement of the symptom by tapering the immunosuppressants [23]. In another patient (no. 8), the serum bilirubin level was normalized after rescue transplantation and diarrhea had also improved (stage 3 → stage 1) by day 23 when the patient died of aspergillus pneumonia. The remaining patient (no. 14) showed a complete response of cutaneous and gut GVHDs, but serum bilirubin levels continued to increase. The aggravation of jaundice was diagnosed to be caused by thrombotic thrombocytopenic purpura based on the presence of severe hemolysis and renal failure. In four patients who rejected rescue donor grafts, one patient (no. 11) showed a complete response of cutaneous and intestinal GVHDs, but showed a progressive increase in serum bilirubin levels and died on day 33. The patient was diagnosed from autopsied liver samples with hepatic veno-occlusive disease with no evidence of GVHD. Patient no. 7 achieved a partial response (stage 4 → stage 1) of cutaneous GVHD but died of pneumonia on day 76. Patient no. 12 showed a complete response for cutaneous and intestinal GVHDs, but showed no response of hepatic GVHD, and died of aggravated GVHD on day 46. The remaining patient (no. 10–1) showed a partial response of cutaneous GVHD and also showed partial improvement of intestinal GVHD by day 5, when diarrhea rebounded and was progressively aggravated; therefore, he underwent a second rescue transplantation, after which he achieved rescue donor engraftment and ultimately had a complete response.

Regarding chronic GVHD, only 1 of the 10 patients who survived for >100 days developed limited-type chronic GVHD (skin lesion).

Adverse effects (Table 4)

CMV antigenemia occurred in 11 of 15 transplants (73.3%). The median peak number of CMV antigen-positive leukocytes was 15.4 per 50,000 white blood cells (15.4/50,000), with a range of 2.8/50,000 to 285.7/50,000. No CMV disease was observed.

Three patients developed bacterial infections: one (no. 7) had fatal pneumonia from *Enterococcus cloacae*, and one (no. 16) had *Escherichia coli* sepsis, and one (no. 15) had sinusitis, all were successfully treated with administration of antibiotics. Two patients developed aspergillus pneumonia: one patient (no. 13) was successfully treated by antibiotics and another patient (no. 8) with a pulmonary aspergillus lesion before rescue transplantation died of

aggravated pneumonia and brain fungal embolism. One patient (no. 14) developed fatal thrombotic thrombocytopenic purpura and one (no. 11) fatal hepatic veno-occlusive disease. One patient (no. 10–1) developed pancreatitis, which was improved by conventional treatment. Ten patients (62.5%) developed liver dysfunction with an increase to more than three times the normal upper limit of the transaminase level. The majority of cases of liver dysfunction were due to steroid- or drug-induced toxicities, and the transaminase level in these patients was normalized after tapering or discontinuation of the causative drugs. Other adverse events are shown in Table 4.

Relapse, cause of death, and overall survival

No patients had recurrence of the original disease. Two patients died early because of a poor performance status at rescue transplantation. Among them, 1 patient (no. 2) had severe GVHD accompanied by sepsis hyperbilirubinemia (10.2 mg/dL), and died of multiorgan failure on day 10. Another (no. 3) developed renal failure after the start of conditioning treatment. Despite receiving hemodialysis, he died of renal failure on day 13.

Overall survival at 6 months and 3 years was 44.6% (95% confidence interval [CI], 19.8–86.8%), and 37.2% (95% CI, 12.4–62.0%), respectively. Patients who achieved rescue donor engraftment showed a significantly improved survival rate compared with those who rejected grafts (log-rank test, $p = 0.013$) (Fig. 2). Six of the eight patients who achieved a complete response survived without any GVHD symptoms or relapse of the original diseases, with a median follow-up of 2128 days (range, 490–3304 days). Two of these patients needed no immunosuppressive agents and the others a small dose of steroids. Two of the patients who achieved a complete response died of cardiac failure on day 831 (no. 6) and of hepatic failure on day 163 (no. 15). Three patients who achieved rescue donor engraftment and who did not achieve a complete response died of multiorgan failure, including thrombotic microangiopathy on day 135 (no. 4), fungal pneumonia on day 23 (no. 8), and thrombotic thrombocytopenic purpura on day 72 (no. 14), as described previously. On the other hand, no long-term survivors were observed in patients who rejected rescue donor grafts. The causes of death for patients who rejected grafts were as described here. Performance status at rescue transplantation was important because no long-term survivors were observed among patients with $\leq 20\%$ Karnofsky performance score.

Discussion

In the present study, we clearly showed that severe, steroid-refractory GVHD was successfully treated by allogeneic SCT using grafts from a second allogeneic donor. The response rate was 80.0% (90.9% for patients achieving engraftment and 50.0% for patients rejecting graft).

Table 4. Adverse events (%)

Infection	bacteria	bacteremia	1 (5.9)
		others	2 (11.8)
	fungus		2 (11.8)*
	virus	cytomegalovirus	0 (0)
		herpes zoster	2 (11.8)
	pneumocystis jiroveci		0 (0)
Hypoxemia			1 (5.9)
Hemorrhagic cystitis			2 (11.8)
Thrombotic thrombocytopenic purpura			1 (5.9)
Thrombotic microangiopathy			2 (11.8)
Venoocclusive disease			1 (5.9)
Pancreatitis			1 (5.9)
Liver dysfunction†			10 (58.8)
Hypertension			4 (23.5)
Aseptic necrosis			2 (11.8)
Cataract			2 (11.8)
Hyperglycemia‡			8 (47.1)
Nephrotoxicity§			1 (5.9)
Insufficiency of adrenal gland			1 (5.9)

*One patient had aspergillus pneumonia before transplantation.

†An increase to > 3 times the normal upper limit of transaminase.

‡Insulin dose of > 30U/day was needed to control blood sugar.

§Nephrotoxicity that needed hemodialysis.

Although patients who were enrolled in the present study had a severe GVHD after HLA-mismatched SCT, which is known to be very difficult to control [26], the overall survival at 6 months and 3 years was 44.6% and 37.2%, respectively. Furthermore, the GVHDs were not only steroid-resistant, but also heavily treated: these patients were refractory to a median of four lines of GVHD-specific treatments (12 patients received tumor necrosis factor blockade, 12 ATG, 11 mycophenolate mofetil, and 9 a pulse therapy of mPSL). The rationale for

graft-versus-GVHD treatment is that allogeneically harmful lymphocytes responsible for GVHD are all eliminated by retransplantation using a second allogeneic graft [11]. In the realization of the graft-versus-GVHD concept, there are two major barriers to be overcome: organ toxicity by conditioning treatment and new development of GVHD by a second allogeneic graft.

Regarding the organ toxicities of conditioning treatment, patients with severe GVHD are in a poor state of health due to GVHD-related organ damage, and therefore cannot

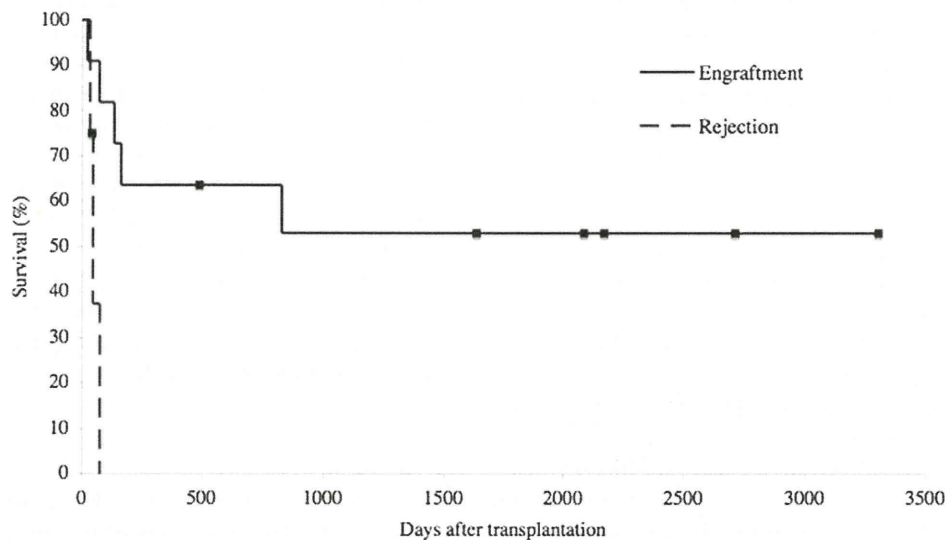


Figure 2. Overall survival of patients with refractory GVHD who did or did not achieve rescue donor engraftment. Patients achieving rescue donor engraftment showed a significantly improved survival rate compared with those rejecting grafts ($p = 0.013$). The survival rate of patients ($n = 11$) who achieved rescue donor engraftment was 63.6% (95% CI, 34.6–92.6%) at 6 months and 53.0% (95% CI, 22.0–84.0%) at 3 years, respectively.