

Figure 1. Receiver-operating characteristic (ROC) curve plot based on the serum C-reactive protein level before first consolidation therapy and the incidence of febrile neutropenia (A) and documented infection (B).

the conventional measurement of the serum CRP level. The slight increase in the serum CRP level might reflect a minute inflammation by occult infection, leading to the overt infectious events during neutropenia.

On the other hand, serum CRP level before the first induction chemotherapy did not have a significant predictive value for infectious events. Serum CRP levels rise in response not only to inflammation, but also to tumour cells [8,9]. Patients before the first induction chemotherapy had a huge number of tumour cells in their bodies, which might have affected the serum CRP level and made it difficult to detect the slight inflammation. In fact, the median serum CRP level just before the first induction chemotherapy was significantly higher than that just before the first consolidation chemotherapy (median 0.3 mg/dl vs

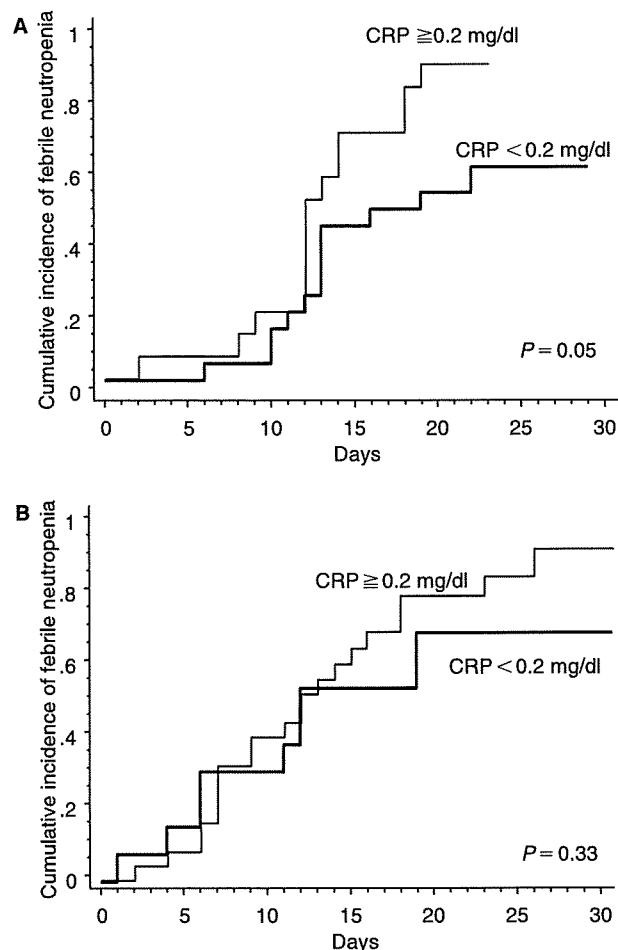


Figure 2. Cumulative incidence of febrile neutropenia grouped according to the serum C-reactive protein (CRP) level before chemotherapy in patients who underwent first consolidation therapy (A) and induction therapy (B).

0.18 mg/dl,  $p = 0.031$ ). This might have reflected the difference in the number of leukaemic cells (average white cell count 22,202/ $\mu$ l vs 4488/ $\mu$ l,  $p = 0.014$  and average blast cell count 15,587/ $\mu$ l vs 14/ $\mu$ l,  $p = 0.0016$ ), although the difference in the CRP level was surprisingly small if we consider the huge difference in the number of leukaemic cells. Another possible explanation is the longer neutropenic duration after induction therapy than consolidation therapy, which increases the possibility of infectious events acquired after starting chemotherapy.

There have been many reports regarding other markers for infectious events. Interleukin-6 (IL-6) is a cytokine that is produced by T-cells or macrophages and regulates the humoral immune response. Procalcitonin (PCT) is a precursor of calcitonin, which is usually produced by thyroid c-cells, but is also produced markedly by monocytes or hepatocytes in response to proinflammatory cytokines. Both IL-6 and PCT have been reported to be useful markers of infection [10,11], especially as markers of bacterial infection [10].

Von Lilienfeld-Toal et al. reported that PCT and IL-6 were more reliable markers than CRP for bacteraemia in patients with FN [12], although Hambach et al. did not confirm the superiority of PCT in allogeneic hematopoietic stem cell transplantation recipients [13]. However, whether IL-6 or PCT has predictive value for infectious events has not been evaluated. If IL-6 and PCT are more specific to inflammation than CRP, the serum levels of IL-6 and PCT, or these factors in combination with CRP, would be more suitable to predict infectious events even during the first induction chemotherapy. This hypothesis should be tested in a future study.

Prophylactic antibiotics are widely administered in patients undergoing chemotherapy. A recent meta-analysis of randomized controlled trials revealed that antibiotic prophylaxis for neutropenic patients, especially with fluoroquinolones, reduced mortality [14]. However, such prophylaxis may lead to the emergence of antibiotic-resistant pathogens. Therefore, it is still important to limit the use of prophylactic antibiotics as much as possible. Identifying low-risk patients by serum inflammatory markers might prevent the excessive use of antibiotics.

In conclusion, serum high-sensitivity CRP levels measured just before consolidation chemotherapy for AML show significant correlation with the development of FN and DI during neutropenia. Therefore, it may become possible to discriminate high-risk patients for infectious events during neutropenia by the CRP level. Further studies are required to establish the appropriate prophylactic methods during neutropenia based on risk stratification according to baseline CRP level.

### Acknowledgements

This research was supported by a Grant-in-Aid for Scientific Research from the Ministry of Health, Labour and Welfare and the Jichi Medical University Young Investigation Award.

**Declaration of interest:** The authors report no conflicts of interest. The authors alone are responsible for the content and writing of the paper.

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## ORIGINAL ARTICLE

# Pharmacokinetics of CsA during the switch from continuous intravenous infusion to oral administration after allogeneic hematopoietic stem cell transplantation

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We investigated the serial changes in the blood CsA concentration during the switch from continuous intravenous infusion to twice-daily oral administration in allogeneic hematopoietic stem cell transplant recipients ( $n = 12$ ). The microemulsion form of CsA, Neoral, was started at twice the last dose in intravenous infusion in two equally divided doses. The area under the concentration–time curve during oral administration ( $AUC_{PO}$ ) was significantly higher than the AUC during intravenous infusion ( $AUC_{IV}$ ) (median 7508 vs 6705 ng/ml  $\times$  h,  $P = 0.050$ ). The median bioavailability of Neoral, defined as ( $AUC_{PO}/DOSE_{PO}$ ) divided by ( $AUC_{IV}/DOSE_{IV}$ ), was 0.685 (range, 0.45–1.04). Concomitant administration of oral voriconazole ( $n = 4$ ) significantly increased the bioavailability of Neoral (median 0.87 vs 0.54,  $P = 0.017$ ), probably due to the inhibition of gut CYP3A4 by voriconazole. Although the conversion from intravenous to oral administration of CsA at a ratio of 1:2 seemed to be appropriate in most patients, a lower conversion ratio may be better in patients taking oral voriconazole. To obtain a similar AUC, the target trough concentrations during twice-daily oral administration should be halved compared with the target concentration during continuous infusion.

*Bone Marrow Transplantation* advance online publication, 9 November 2009; doi:10.1038/bmt.2009.316

**Keywords:** CsA; pharmacokinetics; bioavailability; drug interaction

## Introduction

CsA is the most widely used immunosuppressive agent for the prophylaxis of GVHD after allogeneic hematopoietic

stem cell transplantation (HSCT). It is usually administered by intravenous infusion for at least several weeks after allogeneic HSCT because of the damage done to the oral and gastrointestinal mucosa by the conditioning regimen. However, the dose, target blood level, and schedule of administration vary among protocols and have not been optimized.<sup>1</sup> It has been shown that the blood concentration of CsA affects the incidences of acute GVHD and adverse events,<sup>2</sup> and an increase in the target blood concentration from 300 to 500 ng/ml in the continuous infusion of CsA significantly decreased the incidence of acute GVHD.<sup>3</sup> On the basis of these results, we are currently administering CsA by continuous infusion with target concentrations of 500 ng/ml for standard-risk patients and 300 ng/ml in high-risk patients. When patients can tolerate oral intake, CsA is switched from intravenous to oral administration at a dose ratio of 1:2. Neoral, a microemulsion formulation of CsA, has improved bioavailability and is the most commonly used oral product.<sup>4</sup> However, the appropriateness of this conversion rate has been inconsistent among earlier studies.<sup>5,6</sup> Parquet *et al.* reported that doubling the last intravenous dose provided the best therapeutic range concentration, whereas the concentration/dose ratio was similar in intravenous administration and oral administration and thus, 1:1 conversion seemed appropriate in the McGuire's study. In addition, no data are available regarding the detailed pharmacokinetics in allogeneic HSCT recipients. Therefore, in this study, we investigated the serial changes in the CsA blood concentration during the switch from intravenous to oral administration and assessed the bioavailability of Neoral.

## Patients and methods

### Patients

Patients who underwent allogeneic HSCT with GVHD prophylaxis consisting of the continuous infusion of CsA and short-term MTX were included. This single-center prospective study was approved by the Institutional Review Board of Jichi Medical University, and each patient provided their written informed consent to be enrolled in the study.

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Received 22 June 2009; revised 17 September 2009; accepted 29 September 2009

### Transplantation procedure

The conditioning regimen was mainly a combination of cyclophosphamide (60 mg/kg for 2 days) and TBI (2 Gy twice daily for 3 days) ( $n=8$ ). Patients with severe aplastic anemia ( $n=3$ ) were prepared with fludarabine, cyclophosphamide, and anti-thymoglobulin with or without a low dose of TBI at 2 Gy.<sup>7</sup> A reduced-intensity regimen with fludarabine and melphalan was used for a 58-year-old patient with acute lymphoblastic leukemia ( $n=1$ ). GVHD prophylaxis consisted of the continuous infusion of CsA with a starting dose of 3 mg/kg/day and short-term MTX (10–15 mg/m<sup>2</sup> on day 1 and 7–10 mg/m<sup>2</sup> on days 3 and 6, and optionally on day 11 in HSCT from a donor other than an HLA-matched sibling). The dose of CsA was adjusted to maintain the blood CsA concentration between 450 and 550 ng/ml in standard-risk patients ( $n=9$ ) or 250 and 350 ng/ml in high-risk patients ( $n=3$ ) according to the disease status.<sup>3</sup> Acute GVHD was graded as described earlier.<sup>8</sup> Prophylaxis against bacterial, fungal, and *Pneumocystis jirovecii* infection consisted of levofloxacin, fluconazole (FLCZ), and sulfamethoxazole/trimethoprim (ST) or inhalation of pentamidine. In three patients, micafungin (MCFG) was used instead of FLCZ because of persistent fever despite broad-spectrum antibiotic therapy, development of Candidemia, and high risk for invasive aspergillosis, respectively. As prophylaxis against herpes simplex virus infection, acyclovir (ACV) was given from days -7 to 35, followed by a long-term low-dose administration of ACV for varicella zoster reactivation.<sup>9</sup> Pre-emptive therapy with ganciclovir for cytomegalovirus infection was performed by monitoring cytomegalovirus antigenemia.<sup>10</sup>

### Study schedule

When patients were able to tolerate oral intake, CsA was switched from continuous infusion to oral administration. Intravenous infusion was stopped just before the first oral administration. The initial dose of Neoral was twice the last daily dose of continuous infusion, and was given in two equally divided doses based on the reported bioavailability of Neoral of about 0.4 (40%) in allogeneic HSCT recipients.<sup>5</sup> On the last day of the continuous infusion of CsA (day -1), the serum CsA concentration was measured at 9:00, 15:00, and 21:00. After the patient was switched to Neoral, the CsA concentration was measured just before (C<sub>0</sub>), and 1 (C<sub>1</sub>), 2 (C<sub>2</sub>), 3 (C<sub>3</sub>), 4 (C<sub>4</sub>), 6 (C<sub>6</sub>), and 12 (C<sub>12</sub>) hours after the oral administration of Neoral on the first day (day 0) and between day 3 and day 5. The CsA concentration was measured using the CYCLO-Trac SP-whole blood kit (DiaSorin, Inc., Stillwater, MN, USA).<sup>11</sup> In brief, 200  $\mu$ l of whole blood sample was mixed with 800  $\mu$ l of methanol and centrifuged at 1600 g for 5 min. The methanolic supernatant (50  $\mu$ l in duplicate) was mixed with 100  $\mu$ l of <sup>125</sup>I-ligand and 1 ml of anti-CYCLO-Trac Immune Sep (pre-mixed mouse monoclonal antibody, donkey anti-mouse serum, and normal mouse serum). After centrifuging, the ligand was discarded by decanting and the amount of radioactivity of the pellet was determined. Data were analyzed by logit-log reduction. The standard curve was obtained using the CsA standard sera provided in the kit. The intra-assay coefficient of variance was <15%. The

inter-assay coefficient of variance was <14%. The limit of detection was 4.0 ng/ml. The results of this assay showed good correlation with those obtained by high-performance liquid chromatography ( $r=0.98$ ).

During the study, the dose of CsA could be modified at the discretion of each physician. Vital signs and laboratory variables including renal and liver function tests were evaluated on days 0, 3, 7, and 14. Concomitant medications that could potentially interact with CsA were recorded.

### Statistical considerations

The area under the concentration-time curve (AUC) (0–12 h) of CsA was calculated by the trapezoidal method. We estimated the bioavailability of Neoral by dividing (AUC<sub>PO</sub>/DOSE<sub>PO</sub>) by (AUC<sub>IV</sub>/DOSE<sub>IV</sub>). Toxicities after switching from intravenous to oral administration were evaluated compared with the baseline data on day 0. Renal toxicity was defined as an elevation of the creatinine (Cr) level above  $\times 1.5$  the baseline value. Liver dysfunction was defined as an elevation of alanine aminotransferase (ALT) above  $\times 2$  the baseline value, or elevation of the total bilirubin (T-bil) level by 2 mg per 100 ml compared with the baseline value. Comparisons were made using the Wilcoxon signed-rank test for continuous variables. The Pearson correlation coefficient was used to analyze the correlation between AUC and the CsA concentration at each measurement point after logarithmic transformation. The effect of concomitant medications on CsA pharmacokinetics was first analyzed by a univariate analysis with the Mann-Whitney *U*-test, and then those with at least borderline significance ( $P<0.10$ ) were subjected to a multivariate analysis using multiple regression modeling. A *P*-value of <0.05 was considered to be significant.

## Results

### Patients

Between January 2008 and April 2009, 12 patients were enrolled in the study. There were 7 males and 5 females with a median age of 34.5 years (range, 16–58). Underlying diseases included acute myeloblastic leukemia ( $n=4$ ), acute lymphoblastic leukemia ( $n=3$ ), severe aplastic anemia ( $n=3$ ), chronic myelogenous leukemia ( $n=1$ ), and myelodysplastic syndrome ( $n=1$ ). Five patients received bone marrow graft from an unrelated donor, whereas 1 and 6 patients, respectively, received bone marrow and peripheral blood stem cell graft from a related donor. There was an HLA mismatch in three donor-recipient pairs.

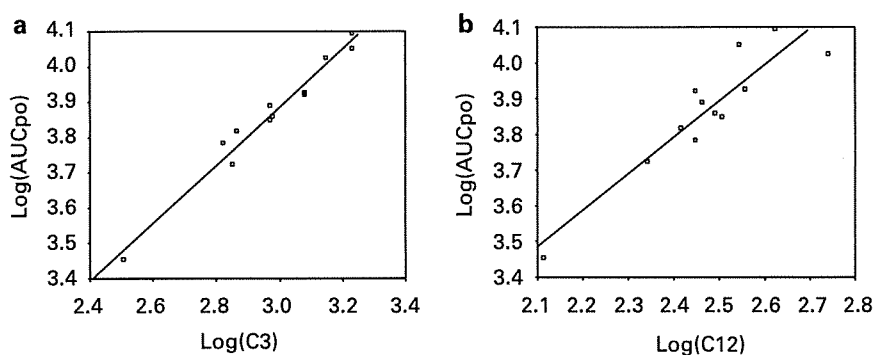
### Pharmacokinetic analysis

The median duration from transplantation to the switch from intravenous to oral administration was 40 days (range, 27–60). The dose of CsA and the pharmacokinetic parameters during intravenous and oral administration are shown in Table 1. Neoral was started at approximately twice the last dose of intravenous infusion, except that 1 patient (No. 8) received Neoral at the same dose as in intravenous infusion, as the mean CsA concentration on the last day of intravenous infusion was >700 ng/ml.

**Table 1** Dose of CsA and pharmacokinetic parameters during the intravenous and oral administration of CsA

Patient no.	Day -1			Day 0					Steady state (Days 3-5)				
	DOSE <sub>IV</sub> (mg/day)	C <sub>mean</sub> (ng/ml)	AUC <sub>IV</sub> (ng/ml × h)	DOSE <sub>PO</sub> (mg/day)	C <sub>max</sub> (ng/ml)	T <sub>max</sub> (h)	C <sub>min</sub> (ng/ml)	AUC <sub>IV-PO</sub> (ng/ml × h)	DOSE <sub>PO</sub> (mg/day)	C <sub>max</sub> (ng/ml)	T <sub>max</sub> (h)	C <sub>min</sub> (ng/ml)	AUC <sub>PO</sub> (ng/ml × h)
1	96	590	7110	200	1300	2	370	9525	160	1400	3	550	10 625
2	140	643	7680	280	1600	3	480	10860	250	1000	2	320	7080
3	130	553	6630	260	2700	3	360	12555	160	1200	2	290	7790
4	173	663	7950	360	1900	2	340	11785	360	2500	1	420	12 420
5	192	677	7920	400	1500	3	240	8685	400	1500	2	280	8355
6	125	577	6780	260	1200	2	360	8300	260	1200	3	360	8450
7	80	527	6330	160	650	0	390	5725	160	800	2	280	6105
8	192	717	8730	200	930	2	360	8100	200	990	4	300	7225
9	240	477	5820	500	1600	3	280	9035	500	2400	2	290	11 265
10	125	357	4350	260	840	2	210	5285	260	880	2	210	5310
11	58	257	3090	120	720	2	130	3375	120	360	4	110	2860
12	77	303	3690	160	1100	2	190	6025	160	1000	1	260	6590

Abbreviations: AUC<sub>IV</sub>=area under the concentration-time curve (AUC) during continuous infusion; AUC<sub>PO</sub>=AUC during oral administration; DOSE<sub>IV</sub>=dose of CsA during continuous infusion; DOSE<sub>PO</sub>=dose of CsA during oral administration.



**Figure 1** Correlation between the AUC and the CsA peak (a: C<sub>3</sub>) and trough (b: C<sub>12</sub>) levels.

In three patients (Nos. 1, 2, and 3), the dose of CsA was reduced on day 1 due to the high CsA concentration on day 0 (the day when Neoral was started).

The median AUC value was 6705 ng/ml × h (AUC<sub>IV</sub>; range, 3090–8730) before the conversion from intravenous to oral administration (day -1), 8493 ng/ml × h (AUC<sub>IV-PO</sub>; range, 3375–12 555) on day 0, and 7508 ng/ml × h (AUC<sub>PO</sub>; range, 2860–12420) on days 3–5, respectively. AUC<sub>PO</sub> was considered to be the AUC of Neoral in the steady state, as AUC<sub>IV-PO</sub> was affected by the intravenous administration of CsA and at least 3 days are required for the CsA concentration to stabilize after a change in the administration route. As a result, not only AUC<sub>IV-PO</sub> but also AUC<sub>PO</sub> was significantly higher than AUC<sub>IV</sub> ( $P=0.050$ ), even though the dose of Neoral was reduced in three patients and the conversion ratio was 1:1 in another patient. The median bioavailability of Neoral was 0.685 (range, 0.45–1.04).

#### Relationship between AUC and the CsA concentration at each measurement point

Although the CsA concentration at each measurement point significantly correlated with AUC<sub>PO</sub> after logarithmic transformation, the strongest correlation was observed between C<sub>3</sub> and AUC<sub>PO</sub> (Figure 1a and Table 2, correlation

coefficient 0.984,  $P<0.001$ ). The AUC<sub>PO</sub> could be predicted from the trough concentration (C<sub>0</sub> or C<sub>12</sub>), which is widely measured in daily practice, by the following formula based on the linear regression model:  $\text{Log}(AUC_{PO}) = 1.020 \times \text{Log}(C_{12}) + 1.344$  (Figure 1b). Accordingly, each trough concentration between 50 and 250 ng/ml corresponds to the CsA concentration during the continuous intravenous infusion of CsA with the same AUC, calculated by dividing the predicted AUC by 12, between 99 and 514 ng/ml (Table 3). Thus, when the continuous intravenous administration of CsA with a target concentration of 500 ng/ml was switched to twice-daily oral administration, the target trough level should be about 250 ng/ml to obtain the same AUC. Also, the target blood concentration of 300 ng/ml during continuous infusion corresponds to the target trough concentration at 150 ng/ml during twice-daily oral administration. This estimation was different from that in kidney transplantation by Nakamura et al. (Table 3).<sup>12</sup>

#### Influence of possible confounding factors on the bioavailability of Neoral

With regard to laboratory data, there were no statistically significant correlations between the bioavailability of Neoral and the serum Cr level, ALT level, and T-bil level

**Table 2** Correlation coefficients between the AUC and the cyclosporine concentration at each measurement point

	Correlation coefficient	P-value	Conversion formula
C0	0.869	<0.001	$\text{Log}(\text{AUC}_{\text{PO}}) = 0.846 \times \text{Log}(\text{C}_0) + 1.747$
C1	0.874	<0.001	$\text{Log}(\text{AUC}_{\text{PO}}) = 0.465 \times \text{Log}(\text{C}_1) + 2.539$
C2	0.953	<0.001	$\text{Log}(\text{AUC}_{\text{PO}}) = 0.718 \times \text{Log}(\text{C}_2) + 1.693$
C3	0.984	<0.001	$\text{Log}(\text{AUC}_{\text{PO}}) = 0.821 \times \text{Log}(\text{C}_3) + 1.424$
C4	0.918	<0.001	$\text{Log}(\text{AUC}_{\text{PO}}) = 0.876 \times \text{Log}(\text{C}_4) + 1.319$
C6	0.961	<0.001	$\text{Log}(\text{AUC}_{\text{PO}}) = 1.314 \times \text{Log}(\text{C}_6) + 0.258$
C12	0.921	<0.001	$\text{Log}(\text{AUC}_{\text{PO}}) = 1.020 \times \text{Log}(\text{C}_{12}) + 1.344$

Abbreviation: AUC<sub>PO</sub> = area under the concentration–time curve during oral administration.

**Table 3** Target cyclosporine concentration during continuous infusion to obtain a similar AUC during twice-daily oral administration with each target trough concentration

Trough level of CsA during twice-daily oral administration (ng/ml)	Corresponding CsA concentration during continuous infusion	
	Nakamura et al. <sup>12</sup>	Current study
50	128	99
100	255	202
150	383	305
200	510	409
250	638	514

Abbreviation: AUC = area under the concentration–time curve.

( $P = 0.867$ ,  $P = 0.159$ , and  $P = 0.770$ , respectively). Four patients had developed acute GVHD before the change in the route of CsA administration, but all of them had stage 1 skin GVHD that was successfully controlled by topical steroid. None of the patients had gastrointestinal involvement and thus the influence of gut GVHD on the bioavailability of Neoral could not be evaluated.

With regard to drug interactions, the effects of the following drugs on the bioavailability of Neoral were evaluated; antifungal agents including FLCZ, itraconazole (ITCZ), voriconazole (VRCZ), and MCFG, antibacterial agents including ST, vancomycin, fluoroquinolones (FQ), and cefepime, antiviral agents including ACV and ganciclovir (DHPG), and other drugs including amlodipine, sulpiride, gabapentin, and prednisolone (PSL) (Table 4). FLCZ ( $n = 3$ ), ITCZ ( $n = 3$ ), and VRCZ ( $n = 4$ ) were exclusively administered orally. These agents had been started at least 7 days before the change in the route of CsA administration. By the Mann–Whitney *U*-test, VRCZ, FQ, and ST were shown to have significant effects with at least borderline significance ( $P = 0.048$ ,  $P = 0.061$ , and  $P = 0.100$ , respectively). Among these, only VRCZ was identified as an independent significant factor by a multivariate analysis ( $P = 0.017$ ). The median bioavailability of Neoral in patients taking VRCZ was 0.87 (range, 0.76–1.04), whereas it was only 0.54 (range, 0.45–0.94) in those without VRCZ.

*Clinical course after the change in the route of CsA administration*

One patient (No. 2) developed liver dysfunction with an elevation of ALT from 28 IU/l at baseline to 300 IU/l 2

**Table 4** Clinical and laboratory data at the conversion that could influence the cyclosporine pharmacokinetics

Patient no.	AUC <sub>iv</sub>	AUC <sub>po</sub>	DOSE <sub>iv</sub>	DOSE <sub>po</sub>	Bioavailability		aGVHD		Cr (mg per 100 ml)	Liver function		Concomitant medications	
					Grade	Stage	ALT (IU/l)	T-bil (mg per 100ml)		Antifungal agents	Others		
1	74	66	0.89	0.89	I	0	0	0	1.14	40	0.24	VCM, ST, ACV, PPI	
2	55	28	0.51	0.51	0	0	0	0	0.65	28	0.9	ACV, PPI, FQ	
3	47	49	1.04	1.04	0	0	0	0	0.81	182	0.77	ST, ACV, PPI, amlodipine gabapentin	
4	46	35	0.76	0.76	I	0	0	0	0.98	28	1.06	ST, ACV, PPI, PSL	
5	41	21	0.51	0.51	0	0	0	0	0.89	43	0.33	ACV, PPI	
6	54	33	0.61	0.61	0	0	0	0	0.65	92	0.79	DHPG, PPI, amlodipine	
7	79	38	0.48	0.48	I	0	0	0	0.72	85	0.59	DHPG, PPI, amlodipine	
8	45	36	0.8	0.8	0	0	0	0	0.60	78	0.78	ACV, PPI	
9	24	23	0.94	0.94	0	0	0	0	0.66	96	0.65	MCFG, ACV, PPI, amlodipine	
10	35	20	0.57	0.57	0	0	0	0	1.43	46	0.37	CFPM, ACV, PPI	
11	53	24	0.45	0.45	I	0	0	0	0.84	16	0.53	CFPM, ACV, PPI	
12	48	41	0.85	0.85	0	0	0	0	1.19	20	0.55	ACV, PPI, FQ, sulpiride	

Abbreviations: ACV = acyclovir; ALT = alanine aminotransferase; AUC<sub>iv</sub> = area under the concentration–time curve (AUC) during continuous infusion; AUC<sub>po</sub> = AUC during oral administration; CFPM = cefepime; DHPG = ganciclovir; DOSE<sub>iv</sub> = dose of CsA during continuous infusion; DOSE<sub>po</sub> = dose of CsA during oral administration; FLCZ = fluconazole; FQ = fluoroquinolones; ITCZ = itraconazole; MCFG = micafungin; PPI = proton pump inhibitors; PSL = prednisolone; ST = sulphametoxazole-trimetoprim; VCM = vancomycin; VRCZ = voriconazole.

**Table 5** Serial changes in laboratory data and blood pressure after the change in the route of CsA administration

	Mean (minimum–maximum)			
	Serum creatinine (mg per 100 ml)	ALT (IU/l)	Total bilirubin (mg per 100 ml)	Blood pressure level (mm Hg)
Day 0	0.87 (0.60–1.43)	64.4 (16–182)	0.63 (0.24–1.06)	Systolic 130 (114–173) Diastolic 82 (63–103)
Day 3	0.86 (0.32–1.63)	50.1 (10–106)	0.62 (0.27–1.47)	Systolic 124 (109–150) Diastolic 79 (51–103)
Day 7	0.92 (0.69–1.31)	44.6 (10–103)	0.61 (0.30–1.17)	Systolic 122 (109–132) Diastolic 80 (51–103)
Day 14	0.83 (0.67–1.29)	65.8 (10–300)	0.64 (0.27–0.96)	Systolic 121 (113–135) Diastolic 76 (68–89)

Abbreviation: ALT = alanine aminotransferase.

weeks after the conversion. The AUC of CsA was rather lower after conversion, and thus CsA was not considered to be the causative agent of liver dysfunction. Otherwise, no notable changes in laboratory and clinical data were observed (Table 5).

Four patients had developed grade I acute GVHD of the skin before the change in the route of CsA administration. During the 2 weeks after the switch, 3 of the 4 patients had persistent grade I skin GVHD, whereas GVHD was improved in 1 patient. Among the eight patients who did not have acute GVHD at the switch, one patient developed grade I acute GVHD of the skin, which was well controlled by topical steroid, and the other seven patients did not develop acute GVHD during the observation period. No clinically significant changes in vital or biological parameters occurred in the study patients. One patient (No. 9) developed nausea soon after conversion. An excessive increase in the CsA concentration was considered to be the cause of nausea and this symptom was improved after the dose of Neoral was reduced.

## Discussion

Neoral is a microemulsion formulation of CsA that has improved bioavailability and reduced variability in pharmacokinetic parameters within and between patients compared with a conventional CsA formulation (Sandimmun).<sup>4</sup> Its bioavailability has been reported to be 0.38 (38%) in healthy volunteers.<sup>13</sup> However, allogeneic HSCT patients have complications that could influence the CsA pharmacokinetics, such as damaged gastrointestinal mucosa and multiple drug interactions. The results of this study showed that the median value of the bioavailability of Neoral was 0.685 (range, 0.45–1.04). Detailed analyses revealed that the oral administration of VRCZ strongly affected the bioavailability of Neoral (0.87 vs 0.54). Therefore, although the switch from intravenous to oral administration of CsA at a ratio of 1:2 seemed to be appropriate in most patients, a lower conversion ratio such as 1:1.1 or 1:1.2 may be better in patients taking oral VRCZ.

The drug interactions between CsA and azole antifungal agents including FLCZ, ITCZ, and VRCZ have been well recognized.<sup>14</sup> Azole antifungal agents are metabolized through the cytochrome P450-3A (CYP3A4) enzyme system, interfere with the metabolism of CsA, and thereby

increase the exposure to CsA. Therefore, careful monitoring of the blood CsA concentration is recommended when these agents are added during CsA administration. On the other hand, there are considerable differences among azole antifungals with regard to their ability to inhibit CYP3A4.<sup>14</sup> Interestingly, the concomitant use of oral VRCZ significantly increased the bioavailability of Neoral. We confirmed that VRCZ was started at least 7 days before the switch from intravenous to oral administration of CsA and was continued at the same dose after the switch. Therefore, the drug interaction between CsA and VRCZ seemed to be stronger during oral administration than during the intravenous infusion of CsA. We hypothesized that this stronger interaction can be explained by the presence of the P450 enzyme system in the gastrointestinal mucosa. The CYP3A4 isoenzymes are the most abundant isoforms of CYP and it has been postulated that CsA is also metabolized in the intestine by gut CYP3A4 isoenzymes.<sup>15</sup> The administration of VRCZ might have inhibited the gut metabolism of CsA and increased the bioavailability of CsA. However, a prospective controlled study is required to confirm this hypothesis.

ITCZ, another strong inhibitor of CYP3A4, did not increase the bioavailability of Neoral. As the ratio of  $AUC_{IV}/DOSE_{IV}$  was higher not only in patients taking VRCZ but also in patients taking ITCZ compared with other patients (median 47.5, 55, and 41), ITCZ might have inhibited liver CYP3A4 similar to VRCZ, but inhibited gut CYP3A4 less strongly than VRCZ. This might have been affected by the different bioavailable dose of these agents, as the bioavailability of ITCZ is lower than that of VRCZ, in addition to the fact that the dose of ITCZ was lower than that of VRCZ (200 vs 400 mg/day).

With regard to the route of VRCZ, it was exclusively administered orally in this study. Therefore, we could not conclude whether the intravenous administration of VRCZ would similarly affect the bioavailability of CsA. In earlier reports, the extent of drug interaction between CsA and azole antifungals varied according to the route of administration and the dose or kind of antifungal agent. Numerous reports have shown a significant interaction (>84%) between oral FLCZ with a dose of 200 mg/day or greater and oral CsA.<sup>16,17</sup> On the other hand, Osowski *et al.*<sup>18</sup> evaluated the drug interaction between intravenous FLCZ at 400 mg/day and intravenous CsA in HSCT recipients and there was a statistically significant but smaller increase (21%) in the serum CsA concentration.

Mihara *et al.*<sup>19</sup> reported that the mean steady-state whole-blood level of CsA significantly increased after the route of FLCZ administration was switched from intravenous to oral. These data suggest that the drug interaction between CsA and FLCZ was stronger when FLCZ was administered orally. With regard to other azole antifungal agents, not only oral but also intravenous administration of ITCZ significantly affected the blood concentration of CsA.<sup>20–22</sup> Concerning the interaction between VRCZ and CsA, Mori *et al.*<sup>23</sup> reported that the administration of VRCZ to patients receiving CsA resulted in a significant increase in the concentration/dose ratio of CsA, but the route of VRCZ administration did not affect the changes in the concentration/dose ratio. If we consider these findings together, it may be reasonable to suggest that the interaction between azole antifungal agents and CsA is stronger when the antifungals are given orally, but the difference becomes unclear with ITCZ and VRCZ, as the interactions of these agents are stronger than that of FLCZ and can be detected even when they are given intravenously. Therefore, when we interpret pharmacokinetic data of CsA, we must be cautious not only about concomitantly used agents but also the route of administration of both CsA and the other drugs. For example, Parquet *et al.* reported that a ratio of 1:2 in the switch from intravenous to oral administration was appropriate,<sup>5</sup> whereas a 1:1 ratio seemed to be appropriate in the study by McGuire *et al.*<sup>6</sup> In the former study, oral FLCZ was used concomitantly and thus their conclusion was consistent with our data. In the latter study, information on the use of antifungal agents was not described, and thus the data were difficult to interpret.

When we switch the route of CsA administration from continuous infusion to twice-daily oral administration, the target blood concentration should also be changed. Nakamura *et al.*<sup>12</sup> reported that the CsA blood concentration during continuous infusion was estimated to be 2.55 times the trough level during twice-daily oral administration of Neoral to obtain an equal AUC of CsA in kidney transplant patients. In this study, we concluded that the CsA concentration during continuous infusion should be doubled compared with the trough concentration during twice-daily oral administration in allogeneic HSCT recipients. Although the calculation method was different, the conclusion was consistent (mean 2.01) when we applied their methods. Although the reason for the difference between these studies remains unclear, it may have been due to the differences in the use of concomitant drugs or the status of the gastrointestinal tract.

In conclusion, when switching CsA from continuous infusion to oral administration, concomitant medications that could affect the bioavailability of CsA, especially azole antifungal agents, should be taken into account. Although a 1:2 ratio on switching may be appropriate in most patients, a lower conversion ratio is recommended in patients taking oral VRCZ.

#### Conflict of interest

The authors declare no conflict of interest.

#### Acknowledgements

This research was supported in part by grants from the Ministry of Health, Labor and Welfare of Japan.

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## ORIGINAL ARTICLE

# A randomized controlled trial of plasma real-time PCR and antigenemia assay for monitoring CMV infection after unrelated BMT

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Preemptive therapy is the standard strategy for preventing CMV disease after allogeneic hematopoietic SCT. In this study, unrelated BMT recipients were randomly assigned to a plasma real-time PCR group or an antigenemia group to compare the value of these monitoring tools for CMV reactivation. Ganciclovir (GCV) was started at 5 mg/kg/day when PCR reached 300 copies per ml or when antigenemia reached three positive cells per two slides. A total of 88 patients were randomized into the antigenemia group ( $n = 45$ ) or the PCR group ( $n = 43$ ). A significantly higher number of patients reached the threshold in the antigenemia group than in the PCR group (73.3 vs 44.2%,  $P = 0.0089$ ). However, only three patients (one in the antigenemia group and two in the PCR group) developed early CMV disease. These patients exclusively had colitis and were successfully treated with GCV or foscarnet. The median number of antigenemia-positive cells at the start of GCV was 47 in the PCR group. These findings suggest that antigenemia assay with the current cutoff was too sensitive and led to unnecessary use of GCV. However, the appropriateness of the threshold may be different by the methodology used, and therefore, it is difficult to generalize.

*Bone Marrow Transplantation* advance online publication, 7 December 2009; doi:10.1038/bmt.2009.337

**Keywords:** CMV; antigenemia; real-time PCR; preemptive therapy

## Introduction

Cytomegalovirus infection is a frequent complication after allogeneic hematopoietic SCT. Universal prophylaxis with ganciclovir (GCV) did not improve the transplantation outcome because of neutropenia caused by GCV.<sup>1,2</sup> Therefore, the initiation of GCV triggered by the detection of CMV reactivation is currently the standard strategy for preventing CMV disease.<sup>3–5</sup> A CMV antigenemia assay has been widely used to monitor CMV reactivation. However, the details of preemptive therapy still need to be clarified, including the threshold number of antigenemia-positive cells for deciding when to start GCV, the dose and duration of GCV and so on. We previously showed that a risk-adapted preemptive therapy, in which the cutoff number of antigenemia-positive cells for deciding when to start GCV was changed according to the risk for CMV disease, was appropriate in allogeneic SCT recipients, but the incidence of neutropenia was still high.<sup>6</sup> Therefore, in the next study, we evaluated the feasibility of preemptive therapy with low-dose GCV, and the findings showed that the initial dose of GCV could be safely decreased to 5 mg/kg.<sup>7</sup>

The PCR used to detect CMV DNA has also been investigated for its ability to monitor CMV reactivation.<sup>8</sup> PCR using whole blood samples might be too sensitive as a trigger for deciding when to start preemptive therapy compared with an antigenemia assay or PCR using plasma samples.<sup>9,10</sup> However, the recent development of real-time PCR has enabled the quantification of CMV DNA. Several studies have shown the feasibility of preemptive therapy guided by real-time PCR monitoring using either whole blood or plasma samples.<sup>11–14</sup> As for whole blood real-time PCR, Gerna *et al.* performed two randomized controlled trials of PCR and antigenemia, one in young patients (0–25 years old) and the other in older patients (20–67 years old).<sup>12,13</sup> They showed that a threshold value of 10 000

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Received 1 July 2009; revised 1 October 2009; accepted 12 October 2009

copies per ml for determining when to start GCV by whole blood PCR significantly reduced the use of GCV compared with a threshold in which GCV is started at any level of positive antigenemia. However, the study included heterogeneous patients in terms of donor type, stem cell source and GVHD prophylaxis. In particular, antithymocyte globulin was used in approximately half of the patients, and this may have strongly affected the incidence of CMV reactivation and disease.<sup>15,16</sup> In addition, preemptive therapy guided by antigenemia assay could be more appropriately performed by using a cutoff based on the number of positive cells.

Therefore, we performed a randomized controlled trial of plasma real-time PCR with a cutoff of 300 copies per ml and an antigenemia assay with a cutoff of three positive cells per two slides in a homogenous population of unrelated BMT recipients who received GVHD prophylaxis with a calcineurin inhibitor and MTX.

## Patients and methods

### Patients

Patients were eligible for the study if they were between 20 and 55 years old, would undergo BMT without *in vivo* or *ex vivo* T-cell depletion from an HLA-matched unrelated donor using a myeloablative conditioning regimen and had a good performance status without significant organ dysfunction, as defined in the protocol. Either the donor, the recipient or both must have been seropositive for CMV. Prophylaxis against GVHD was limited to a combination of CYA and MTX, but a combination of tacrolimus and MTX was allowed after June 2002. Patients were enrolled before starting a conditioning regimen, but randomization was performed between day 10 and day 12 after transplantation to exclude patients who developed significant organ dysfunction early after transplantation. This study was approved by the institutional review board of each participating center and a written informed consent was obtained from each patient (UMIN-CTR C000000347).

### CMV monitoring methods

Cytomegalovirus antigenemia assay was performed as described previously.<sup>17</sup> In brief,  $1.5 \times 10^5$  peripheral blood leukocytes were attached to a slide using a cytocentrifuge and fixed with formaldehyde. The cells were sequentially immunostained with MoAb C10/11 (Clonab CMV; Biotest, Dreieich, Germany) and reacted with goat alkaline phosphatase-labeled anti-mouse Ig (Mitsubishi Kagaku Iatron Inc, Tokyo, Japan). Under a light microscopy, CMV-positive cells were counted and the results are presented as the sum of the number of positive cells per two slides.

Real-time PCR was performed using primers and a TaqMan probe for immediate early genes using serum samples.<sup>18</sup> Briefly, DNA extracted from 100  $\mu$ l of plasma was subjected to PCR using TaqMan Universal PCR Master Mix (PE Biosystems, Foster City, CA, USA) and the PCR product was detected as an increase in the

fluorescent intensity using ABI Prism 7700 (PE Biosystems). Real-time fluorescent measurements were taken and a threshold cycle (CT) value for each sample was calculated by determining the point at which the fluorescence exceeded 10 times the baseline fluorescence. A standard curve was constructed using the CT values obtained from serially diluted DNA extracted from a plasmid that contains the respective region of CMV. The CT values from the clinical samples were plotted on the standard curve and the copy number was calculated automatically using Sequence Detection System version 1.6 (PE Biosystems).

### Preemptive therapy against CMV disease

Patients were randomly assigned to the antigenemia group or the PCR group using a random block design. Assignment was stratified by the institute, age and the presence or absence of GVHD at the time of randomization. CMV reactivation was monitored weekly by both the antigenemia assay and PCR in all patients, but only the results of the assigned monitoring method were returned to the physicians. Preemptive therapy with GCV was started at an induction dose of 5 mg/kg/day when three or more CMV-positive cells per two slides were detected in the antigenemia group and 300 or more CMV DNA copies per ml were detected in the PCR group. The dose of GCV was increased to 10 mg/kg/day when a rising CMV load was observed. The dose of GCV was decreased to 5 mg/kg/day when a declining CMV load was observed in patients who were receiving GCV at 10 mg/kg/day. A rising and declining CMV load was defined as an increase and decrease in the CMV load by 50% or more of the previous value, respectively. However, changes in antigenemia-positive cells by less than five cells per two slides and changes in the DNA copy number by less than 500 copies per ml were regarded as a stable CMV load. When the CMV load fell below the threshold to start GCV, the dose of GCV was decreased to 5 mg/kg/day, if the patient was receiving GCV at 10 mg/kg/day, and GCV was discontinued if the patient was receiving GCV at 5 mg/kg/day. The dose of GCV was adjusted according to the renal function.<sup>19</sup> CMV monitoring was continued until all of the following three requirements were fulfilled: (i) More than 100 days had passed after transplantation; (ii) More than 2 weeks had passed after the last administration of GCV; and (iii) Absence of the use of (methyl-)prednisolone at 0.5 mg/kg/day or more.<sup>20</sup>

### Definition of CMV disease

All patients with symptoms compatible with CMV disease such as interstitial pneumonia, colitis and gastritis underwent extensive pathological and microbiological examination of biopsy specimens. The diagnosis of CMV disease was made by histopathological examination and immunohistochemical staining of biopsy specimens. However, CMV retinitis was diagnosed when CMV DNA was detected by PCR using aqueous humor samples associated with characteristic retinal changes by ophthalmoscopy. Early and late CMV diseases were defined as those occurring before and after day 100, respectively.

### Statistical considerations

The primary end point of the study was the incidence of early CMV disease. We defined success as the absence of CMV disease before day 100. Noninferiority was pre-defined as a difference in the success rates between the antigenemia group and the PCR group of no more than 10 percentage points. On the basis of the assumption of a success rate of 95% in the PCR group and 90% in the antigenemia group, 39 patients in each treatment group were required to show noninferiority with an alpha error of 5% and a power of 80%, which permitted a 10% difference in the success rate. On the basis of the assumption of a 20% loss of patients between the enrollment and randomization, a total of 96 patients needed to be enrolled in this study. Comparisons for dichotomous and continuous variables between groups were performed with Fisher's exact test and *t*-test, respectively. Pearson's correlation coefficient was calculated to compare the results of the two monitoring methods after logarithmic transformation.

### Results

#### Incidence of CMV reactivation and the use of GCV

A total of 96 patients were enrolled in the study between January 2002 and March 2007. Among these patients, eight patients were excluded because of the use of tacrolimus as GVHD prophylaxis in one, negative CMV Ab in both the donor and recipient in one and organ dysfunction after the conditioning regimen in six. Therefore, a total of 88 patients were randomized into the antigenemia group (*n* = 45) or the PCR group (*n* = 43) (Figure 1). There were no differences in age, sex, background disease, CMV serostatus, conditioning regimen or GVHD prophylaxis between the two groups (Table 1). In addition, the incidence of grade II–IV acute GVHD was similar (42 vs 47%, *P* = 0.67).

Cytomegalovirus reactivation, defined as a detection of CMV at any level, was more frequently observed in the antigenemia group (40 of 45 patients, 88.9%) than in the

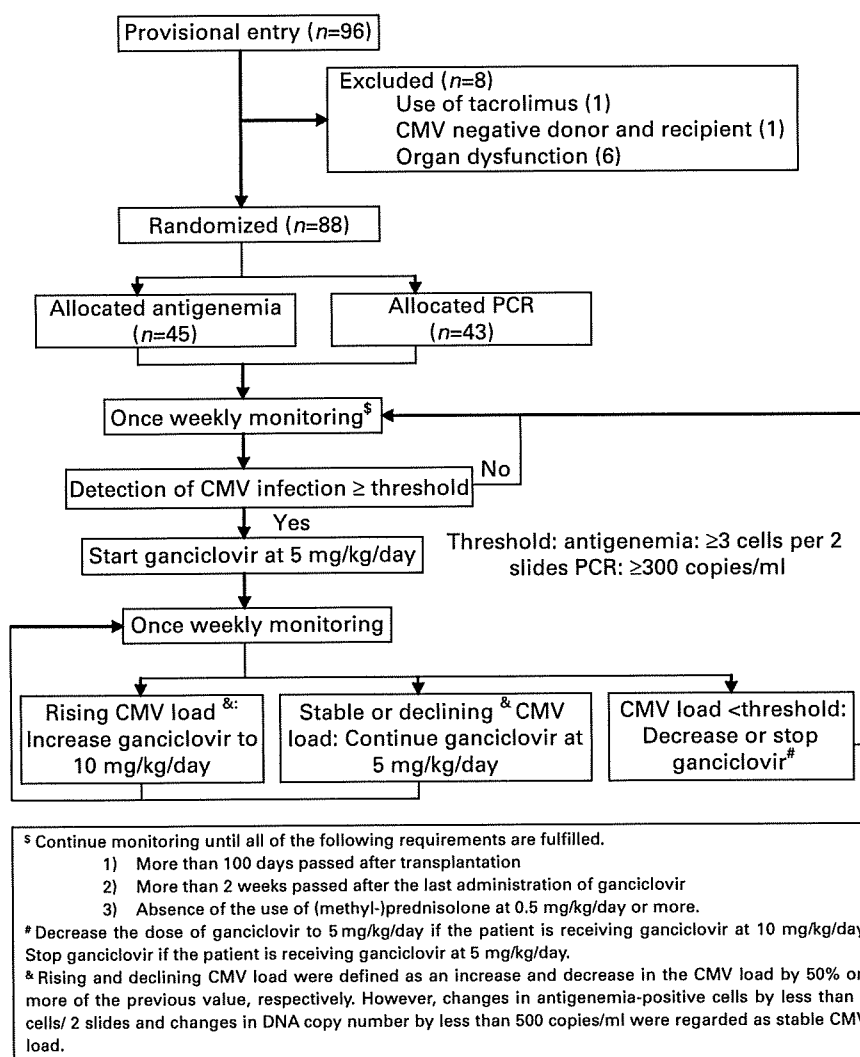


Figure 1 Design of the study.

PCR group (27 of 43 patients, 62.8%) ( $P=0.0050$ , Table 2). The probability of starting GCV was significantly higher in the antigenemia group than in the PCR group (73.3 vs 44.2%,  $P=0.0089$ , Figure 2). The results of PCR in the antigenemia group and those of the antigenemia assay in the PCR group were disclosed after the completion of the study. A good correlation was seen between the results of PCR and the antigenemia assay ( $P<0.0001$ ,  $r^2=0.38$ , Figure 3). Of the 33 patients who received GCV in the antigenemia group, PCR and the antigenemia assay reached the threshold simultaneously in five patients and PCR reached the threshold before starting GCV in only four patients (Figures 4a and 5a). In the other 24 patients, the CMV DNA copy number was persistently below the

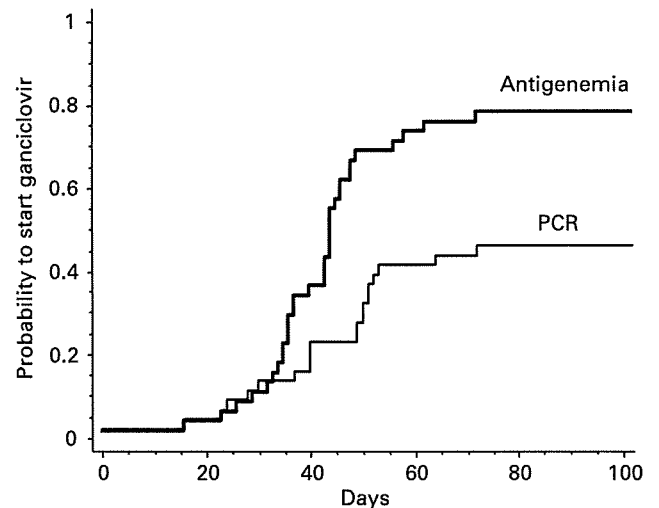
threshold until GCV was started. On the other hand, in 11 of 19 patients who received GCV in the PCR group, the results of the antigenemia assay reached the threshold earlier in 11 patients and simultaneously in 7 patients (Figures 4b and 5b). The results of the antigenemia assay were persistently below the threshold until GCV was started in only one patient. The median number of antigenemia-positive cells at the start of GCV was 5 (range: 3–102) and 47 (range: 0–2921) in the antigenemia and PCR groups, respectively (Figure 6a,  $P=0.0051$ ). The median CMV DNA copy number was negative (range: 0–4400) and 750 (range: 310–13000) in the antigenemia and PCR groups, respectively (Figure 6b,  $P<0.0001$ ).

Among the 52 patients who received preemptive therapy with GCV at 5 mg/kg/day, only 13 and 7 patients in the antigenemia and PCR groups, respectively, experienced a rising CMV load and required dose-escalation to 10 mg/kg/day, suggesting that the initiation of GCV at 5 mg/kg was appropriate.

**Table 1** Patient characteristics

	Antigenemia (n=45)	PCR (n=43)	P-value
<i>Pre-transplantation factors</i>			
Median age (range)	41 (20–55)	40 (20–53)	0.82
Sex (male/female)	25/20	24/19	>0.99
HLA mismatch	7 (16%)	9 (21%)	0.59
<i>Background disease</i>			
AML	17	18	
ALL	12	12	
CML	6	3	
MDS	5	7	
Others	5	3	0.57
<i>Donor/recipient CMV status</i>			
Pos./Pos.	28	26	
Pos./Neg.	5	4	
Neg./Pos.	8	6	0.74
<i>Conditioning regimen</i>			
TBI	39	36	
Non-TBI	6	7	0.77
<i>GVHD prophylaxis</i>			
CYA-MTX	25	25	
TAC-MTX	16	16	0.59

Abbreviations: MDS = myelodysplastic syndrome; Neg. = negative; Pos. = positive; TAC = tacrolimus.



**Figure 2** Days to start ganciclovir after transplantation.

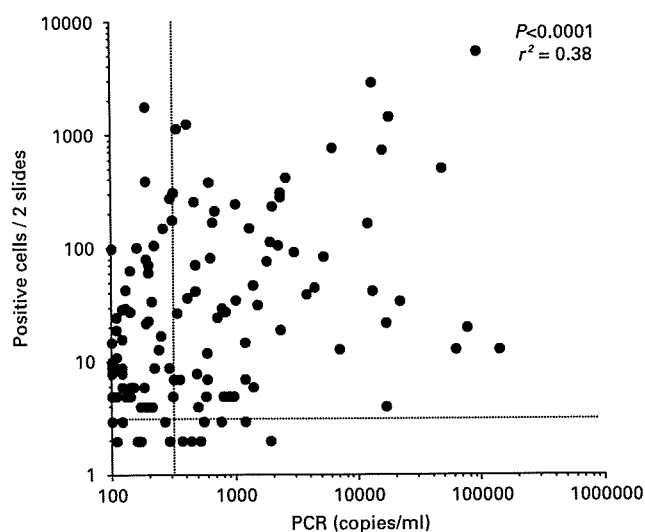
**Table 2** CMV-related events after engraftment

	Antigenemia (n=45)	PCR (n=43)	P-value
CMV reactivation <sup>a</sup>	40	27	0.0050
<i>Start ganciclovir</i>			
Duration of ganciclovir (days)	33	19	0.0089
Duration of ganciclovir (days)	23.2 ± 19.4	20.8 ± 14.2	0.64
Total dose of ganciclovir (mg/kg)	140.8 ± 129.7	118.4 ± 91.2	0.51
Dose escalation to level II	13	7	>0.99
Neutropenia <500 per µl	5	3	>0.99
Stop ganciclovir because of neutropenia	1	0	>0.99
Increase in serum creatinine <sup>b</sup>	8	0	0.039
<i>CMV disease</i>			
Early (before day 100)	1	2	0.61
Late (after day 100)	0	1 <sup>c</sup>	0.48

<sup>a</sup>Detection of antigenemia or DNA at any level.

<sup>b</sup>Increase in serum creatinine level by 0.5 mg per 100 ml or more from the baseline level.

<sup>c</sup>The patient developed early CMV disease, which was improved by ganciclovir. However, intestinal symptoms recurred after day 100 and CMV colitis was suspected because of positive antigenemia, although it was not confirmed by biopsy.



**Figure 3** Correlation between the number of positive cells in the antigenemia assay and copy number by PCR.

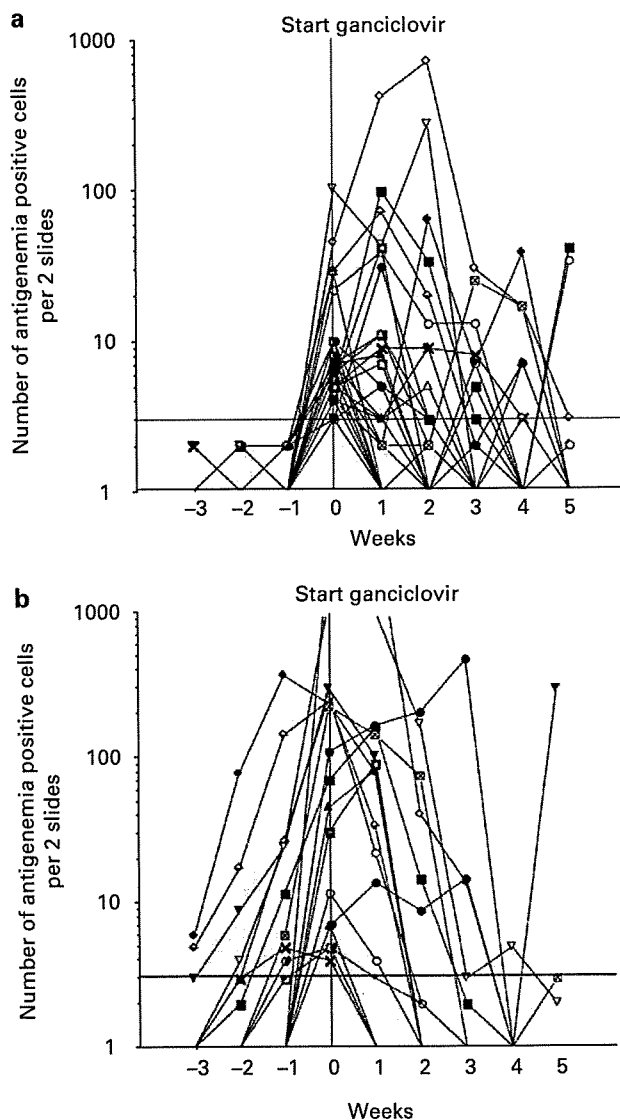
#### CMV diseases

Early CMV disease was diagnosed in 1 of the 45 patients (2.2%) in the antigenemia group and 2 of the 43 patients (4.7%) in the PCR group ( $P=0.61$ ). These patients exclusively developed CMV colitis. Another patient in the PCR group showed characteristic retinal changes and was presumptively treated with GCV, although CMV infection was not detected in either the aqueous humor or the peripheral blood. The 95% confidence interval for the difference in the success rate was  $-10.1$  to  $5.2\%$ , and thus was just outside the predefined lower limit of  $-10\%$ . However, as shown in Table 3, the development of CMV disease in the PCR group could not be avoided even if these patients were assigned to the antigenemia group, as either the antigenemia assay and PCR reached the threshold simultaneously (UPN32) or the antigenemia assay did not reach the threshold before the diagnosis of CMV disease (UPN35). All of these patients were successfully treated with GCV or foscarnet, although one patient (UPN35) showed the recurrence of colitis after day 100. None of the other patients developed late CMV disease.

#### Adverse events during preemptive therapy

The mean duration of preemptive therapy with GCV and the mean total dose of GCV was  $23.2 \pm 19.4$  days and  $140.8 \pm 129.7$  mg/kg in the antigenemia group and  $20.8 \pm 14.2$  days and  $118.4 \pm 91.2$  mg/kg in the PCR group ( $P=0.64$  and  $P=0.51$ ), respectively. Neutropenia with a neutrophil count of  $<500$  per  $\mu\text{l}$  was observed in 5 of the 33 patients in the antigenemia group and 3 of the 19 patients in the PCR group ( $P>0.99$ ). Only one patient in the antigenemia group required a discontinuation of GCV because of neutropenia. The total dose of GCV was higher in patients who developed neutropenia, but this difference was not statistically significant ( $163.8 \pm 82.5$  vs  $126.9 \pm 121.4$ ,  $P=0.42$ ).

An increase in the serum creatinine level by at least  $0.5$  mg per  $100$  ml was observed in 8 of the 33 patients in the antigenemia group and in none of the 19 patients in the

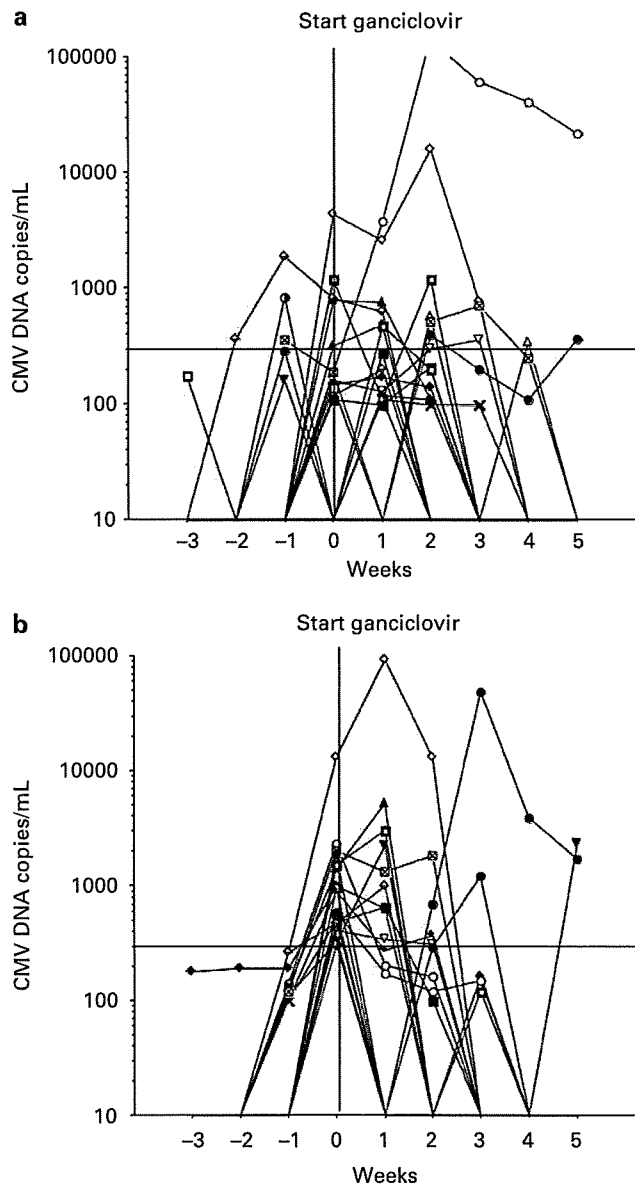


**Figure 4** Serial changes in the number of antigenemia-positive cells in patients who received preemptive therapy in the antigenemia group (a) and in the PCR group (b). Week 0 represents the day ganciclovir was started.

PCR group ( $P=0.039$ ). The total dose of GCV was significantly higher in patients who developed renal impairment ( $255.0 \pm 198.0$  vs  $106.0 \pm 45.5$ ,  $P=0.0004$ ).

#### Discussion

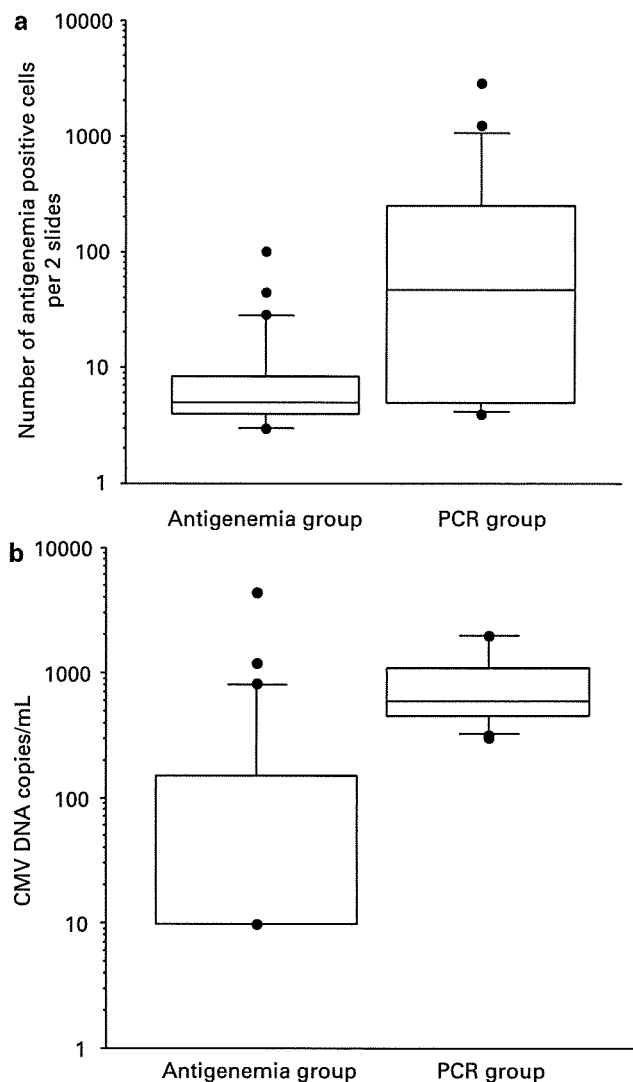
In this randomized controlled trial, we compared plasma real-time PCR with a cutoff at 300 copies per ml and an antigenemia assay with a cutoff at three positive cells per two slides as a trigger for deciding when to start preemptive therapy with GCV after unrelated BMT. GCV was used significantly less frequently in the PCR group. A comparison of the number of antigenemia-positive cells and the CMV DNA copy number at the start of GCV treatment clearly revealed that plasma PCR was significantly less sensitive than the antigenemia assay, at least with the current cutoff values. Although the 95% confidence



**Figure 5** Serial changes in CMV DNA copy number in patients who received preemptive therapy in the antigenemia group (a) and in the PCR group (b). Week 0 represents the day ganciclovir was started.

interval for the difference in the successful prevention rate was just outside the predefined lower limit of  $-10\%$ , and therefore, we could not show the noninferiority of the PCR group, the incidence of CMV disease was limited to two patients even in the PCR group. In addition, prevention of CMV pneumonia, the main aim of preemptive therapy, was completely achieved in both groups. These findings suggest that an antigenemia assay with a cutoff of three positive cells per two slides was too sensitive and resulted in the unnecessary use of GCV.

The unnecessary use of GCV may be reduced if the cutoff value for the antigenemia assay is increased. The antigenemia assay has already been shown to be not sensitive enough for detecting gastrointestinal involvement by CMV



**Figure 6** The number of antigenemia-positive cells (a) and the CMV DNA copy number at the start of preemptive therapy (b), grouped according to the randomization arm. The box-and-whisker plot shows 10, 25, 50, 75 and 90 percentile values. Outliers are indicated by dots.

even with a low threshold.<sup>21</sup> In this study, the median number of antigenemia-positive cells at the start of GCV treatment was 47 in the 19 patients who received preemptive therapy in the PCR group. Figure 7 shows the serial changes in the number of antigenemia-positive cells in the patients of the PCR group who developed positive antigenemia that reached the threshold, but who did not receive GCV at that time. In about half of the patients, antigenemia spontaneously became negative without GCV treatment. On the other hand, seven patients developed high-grade antigenemia of over 100 positive cells per two slides. However, GCV was started when the number of positive cells was 260 (median, range: 73–1262 cells) and none of these patients developed CMV disease. Although patients who developed grade II–IV acute GVHD or who received steroid at 0.5 mg/kg or higher experienced high-grade antigenemia more frequently than those who did not

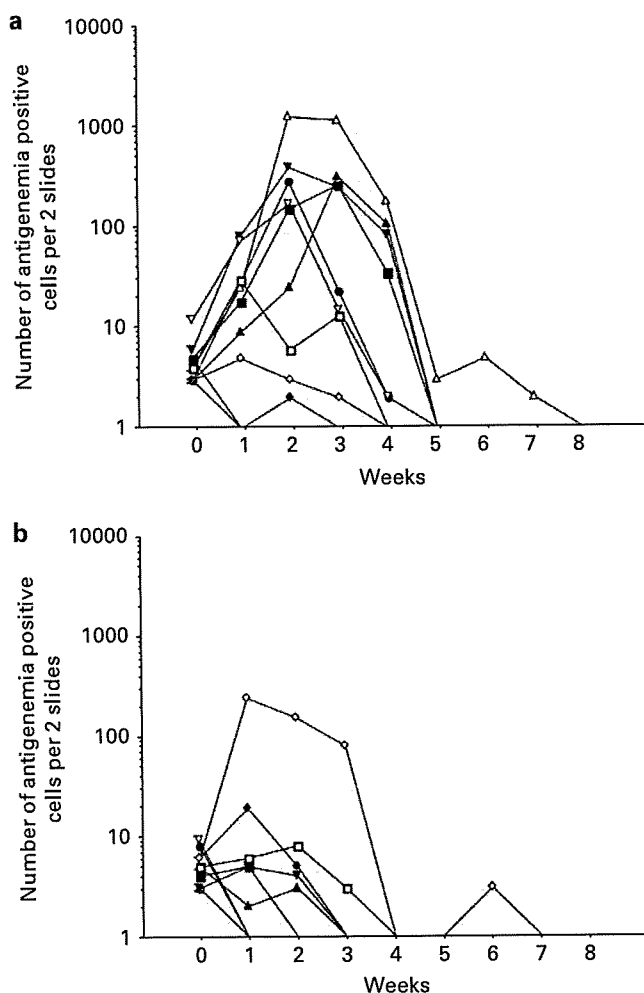
develop grade II–IV acute GVHD and did not receive steroid (Figures 7a and b), the use of GCV was comparable (54.5 vs 40%,  $P=0.67$ ). Thus, although it is difficult to determine the appropriate cutoff value for the antigenemia assay, we thought that it may be worth trying to apply a cutoff value of 20 positive cells per two slides, which we are already safely using in allogeneic hematopoietic SCT from

an HLA-matched sibling donor,<sup>20</sup> to transplantation from an unrelated donor.

Although Boeckh *et al.*<sup>3</sup> reported a 14% incidence of early CMV disease using the same cutoff as in the current study, the incidences of positive antigenemia at any level and three or more positive cells per two slides were similar to those in this study (79 and 70% in Boeckh's study and 89 and 73% in the current study). Therefore, the higher incidence of early CMV disease probably resulted from the high incidence (35%) of grade III–IV acute GVHD in their study rather than from the difference in the method used for the antigenemia assay, as acute GVHD is one of the strongest risk factors for CMV disease.

Nevertheless, it is important to note that the sensitivity and specificity of these assays vary depending on the methodology used.<sup>9,22–24</sup> In fact, the unexpected differences in the sensitivities of the two assays in this study could be explained by the difference in the methodology used in the antigenemia assay. The cutoffs used for the antigenemia assay and real-time PCR were determined based on our previous study in which HRP-C7 Ab was used in the antigenemia assay.<sup>18</sup> In this study, however, we used C10/C11 Ab in the antigenemia assay, as this Ab has been used worldwide. Although we did not believe that there are clinical differences between these two antigenemia assays,<sup>6,7,20</sup> we should have tested the correlation between the results of plasma PCR and the antigenemia assay using C10/C11 Ab. Fortunately, the unexpected difference in the sensitivity in these assays contributed to the finding that the antigenemia assay with the current cutoff was too sensitive as a trigger for deciding when to start preemptive therapy. These data are valid only when the same methodology is used, and standardization of the methods is warranted.<sup>25,26</sup>

In conclusion, CMV colitis could not be completely prevented by the current preemptive strategy using the peripheral blood samples, but CMV pneumonia was completely prevented in both groups. The initiation of GCV at 5 mg/kg/day was confirmed to be safe, provided the CMV load continues to be monitored. Plasma PCR with a cutoff at 300 copies per ml seemed to be appropriate for monitoring CMV reactivation after transplantation. The cutoff number of positive cells should be raised above that used here when using an antigenemia assay. However, the appropriateness of the threshold of these assays may be different on the basis of the methodology and patient background, such as the risk of GVHD, and therefore, it is difficult to generalize.



**Figure 7** Serial changes in the number of antigenemia-positive cells in the PCR group patients who developed positive antigenemia that reached the threshold, but who did not receive ganciclovir. (a) Patients who developed grade II–IV acute GVHD or who received steroid at 0.5mg/kg or more. (b) Patients who did not develop grade II–IV acute GVHD and did not receive steroid.

**Table 3** CMV load in patients who developed CMV disease

Age/sex	Acute GVHD	Onset/affected organ of CMV disease		–3 weeks	–2 weeks	–1 week	Onset
UPN32 38/M (PCR group)	Grade II	Day 56/colitis	PCR	(–)	260	13 000 <sup>a</sup>	93 000
			Ag	(–)	(–)	2921	5467
UPN35 36/M (PCR group)	Grade II	Day 46/colitis	PCR	(–)	(–)	(–)	(–)
			Ag	0	0	2	12
UPN70 38/M (Antigenemia group)	Grade II	Day 50/colitis	PCR	(–)	(–)	110	100
			Ag	2	(–)	5 <sup>a</sup>	99

<sup>a</sup>Preemptive therapy was started.



## Conflict of interest

The authors declare no conflict of interest.

## Acknowledgements

We thank Mrs Aki Tanihara, who was involved in the data management. This research was supported by a Grant-in-Aid for Scientific Research from the Ministry of Health, Labor and Welfare.

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# NKG2D gene polymorphism has a significant impact on transplant outcomes after HLA-fully-matched unrelated bone marrow transplantation for standard risk hematologic malignancies

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## ABSTRACT

**Acknowledgments:** we are indebted to Drs. Hiroko Oshima, Masanobu Oshima and Atsushi Hirao, Mrs. Shinichi Ohmae and Katsuya Nakata, and Ms. Kaori Matsuura at Kanazawa University, and Drs. Keitaro Matsuo and Takakazu Kawase at the Aichi Cancer Center Research Institute for their technical assistance. We thank all of the JMDP transplant teams who contributed patients and donors to this study.

**Funding:** this study was supported by grants from the Ministry of Health, Labor and Welfare, and the Ministry of Education, Culture, Sports and Technology, and funds from the Mitani Research and Development Assistance Organization (Kanazawa, Japan) and by the Japan Leukemia Research Fund (Tokyo, Japan).

Manuscript received on March 5, 2009. Revised version arrived on April 13, 2009. Manuscript accepted on April 29, 2009.

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### Background

NKG2D, an activating and co-stimulatory receptor expressed on natural killer cells and T cells, plays pivotal roles in immunity to microbial infections as well as in cancer immunosurveillance. This study examined the impact of donor and recipient polymorphisms in the *NKG2D* gene on the clinical outcomes of patients undergoing allogeneic T-cell-replete myeloablative bone marrow transplantation using an HLA-matched unrelated donor.

### Design and Methods

The *NKG2D* polymorphism was retrospectively analyzed in a total 145 recipients with hematologic malignancies and their unrelated donors. The patients underwent transplantation following myeloablative conditioning; the recipients and donors were matched through the Japan Marrow Donor Program.

### Results

In patients with standard-risk disease, the donor *NKG2D-HNK1* haplotype, a haplotype expected to induce greater natural killer cell activity, was associated with significantly improved overall survival (adjusted hazard ratio, 0.44; 95% confidence interval, 0.23 to 0.85;  $p=0.01$ ) as well as transplant related mortality (adjusted hazard ratio, 0.42; 95% confidence interval, 0.21 to 0.86;  $p=0.02$ ), but had no impact on disease relapse or the development of grade II-IV acute graft-versus-host disease or chronic graft-versus-host disease. The *NKG2D* polymorphism did not significantly influence the transplant outcomes in patients with high-risk disease.

### Conclusions

These data suggest an association between the donor *HNK1* haplotype and better clinical outcome among recipients, with standard-risk disease, of bone marrow transplants from HLA-matched unrelated donors.

**Key words:** *NKG2D*, *HNK1*, *LNK1*, unrelated donor; bone marrow transplantation, single nucleotide polymorphism.

**Citation:** Espinoza JL, Takami A, Onizuka M, Sao H, Akiyama H, Miyamura K, Okamoto S, Inoue M, Kanda Y, Ohtake S, Fukuda T, Morishima Y, Kodera Y, and Nakao S, for the Japan Marrow Donor Program. *NKG2D* gene polymorphism has a significant impact on transplant outcomes after HLA-fully-matched unrelated bone marrow transplantation for standard risk hematologic malignancies. *Haematologica* 2009;94:1427-1434. doi:10.3324/haematol.2009.008318

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## Introduction

Hematopoietic stem cell transplantation (SCT) is a potentially curative treatment for a range of hematologic malignancies. Although the use of an HLA-matched unrelated donor is well accepted when an HLA-identical sibling donor is unavailable, the risk of transplantation-related complications may be increased.<sup>1</sup> Despite improvements in clinical and supportive care, transplant-related life-threatening complications, including graft-versus-host disease (GVHD), infections and disease relapse, remain an enormous obstacle to overcome.<sup>2</sup> Although HLA matching is the major genetic determinant of clinical outcome after allogeneic SCT, recent evidence suggests that non-HLA immune-associated genes are also implicated.<sup>3</sup> Previous investigations have revealed that several single nucleotide polymorphisms (SNP) which affect individual immune response to infections and inflammatory reactions are associated with the risk of GVHD and transplant outcomes.<sup>4,15</sup>

NKG2D is an activating and co-stimulatory receptor belonging to the C-type lectin-like family of transmembrane proteins and is expressed as a homodimer on natural killer (NK) cells, CD8<sup>+</sup>  $\alpha\beta$ <sup>+</sup> T cells,  $\gamma\delta$ <sup>+</sup> T cells and activated macrophages.<sup>16-18</sup> The ligands for NKG2D, such as MHC class I-chain related proteins (MICA and MICB), UL16 binding proteins are usually absent or expressed at very low levels in normal cells but are up-regulated by cellular stress including heat shock and microbial infections and are frequently expressed in epithelial tumor cells.<sup>19</sup> Ligand engagement of NKG2D triggers cell-mediated cytotoxicity and co-stimulates cytokine production through a DAP10-phosphoinositol 3-kinase dependent pathway and plays an important role in the elimination of tumors and infected cells.<sup>16-18,20</sup>

Recently, SNP were identified between *LNK1* and *HNK1* haplotypes of the *NKG2D* gene.<sup>21</sup> In Japanese individuals, the *HNK1* haplotype is associated with greater activity of NK cells in the peripheral blood<sup>21,22</sup> and a lower prevalence of cancers originating from epithelial cells.<sup>21,23,24</sup> The present study investigates the impact of donor and recipient polymorphisms in the *NKG2D* gene on the clinical outcomes of patients undergoing allogeneic myeloablative bone marrow transplantation using an HLA allele-matched unrelated donor.

## Design and Methods

### Patients

*NKG2D* genotyping was performed on a total 145 recipients with hematologic malignancies and their unrelated donors who were part of the Japan Marrow Donor Program (JMDF). The recipients underwent transplantation, following myeloablative conditioning, with T-cell-replete marrow from an HLA-A, -B, -C, -DRB1 allele-matched donor between November 1995 and March 2000. HLA genotypes of the HLA-A, -B, -C, and -DRB1 alleles of the patients and donors were determined by the Luminex microbead method described previously. (Luminex 100 System; Luminex, Austin, TX, USA).<sup>25,26</sup> No

patient had a history of prior transplantation. The final clinical survey of these patients was completed by November 1, 2007. Diagnoses were acute myeloid leukemia (n=49; 34%), acute lymphoblastic leukemia (n=37; 26%), chronic myeloid leukemia (n=41; 28%), myelodysplastic syndrome (n=11; 8%) and malignant lymphoma (n=7; 5%), (Table 1). The recipients were defined as having standard risk disease if they had acute myeloid or lymphoblastic leukemia in first complete remission, malignant lymphoma in complete remission, chronic myeloid leukemia in any chronic phase or myelodysplastic syndrome. All other patients were designated as having high-risk disease. Myeloid malignancies included acute myeloid leukemia, chronic myeloid leukemia and myelodysplastic syndrome, whereas lymphoid malignancies included acute lymphoblastic leukemia and malignant lymphomas. Cyclosporine or tacrolimus-based regimens were used in all patients for GVHD prophylaxis whereas anti-T-cell therapy, such as anti-thymocyte globulin and *ex vivo* T-cell depletion, was not. All patients and donors gave their written informed consent to molecular studies, according to the declaration of Helsinki, at the time of transplantation. The project was approved by the Institutional Review Board of Kanazawa University Graduate School of Medicine and the JMDF.

### NKG2D genotyping

*NKG2D* was genotyped using the TaqMan-Allelic discrimination method<sup>27</sup> with a 9700-HT real time polymerase chain reaction (PCR) system (Applied Biosystems, Foster City, CA, USA) and results were analyzed using allelic discrimination software (Applied Biosystems). The genotyping assay was conducted in 96-well PCR plates. The amplification reaction contained template DNA, TaqMan universal master mix and a specific probe (product No. C\_9345347\_10; Applied Biosystems) for rs1049174, a single locus featuring a G-C substitution to distinguish between the *HNK1* (G) and *LNK1* (C) haplotypes of the *NKG2D* gene.<sup>21,23,24</sup>

### Data management and statistical analysis

Data were collected by the JMDF using a standardized report form. Follow-up reports were submitted at 100 days, 1 year and annually after transplantation. Pre-transplant cytomegalovirus serostatus was routinely tested only in patients but not in their donors. Engraftment was confirmed by an absolute neutrophil count of more than  $0.5 \times 10^9/L$  for at least 3 consecutive days. Acute and chronic GVHD were diagnosed and graded using established criteria.<sup>28,29</sup> Overall survival was defined as the number of days from transplantation to death from any cause. Disease relapse was defined as the number of days from transplantation to disease relapse. Transplant-related mortality was defined as death without relapse. Any patients who were alive at the last-follow-up date were censored. When collecting data, only the main cause of death was recorded if two or more causes were combined. Data on etiological agents of infections, post-mortem changes and supportive care (including prophylaxis of infections and therapy of GVHD, which were given on an institutional basis), were not available for this

cohort of patients. The analysis was performed using Excel 2007 (Microsoft Corp, Redmond, WA, USA), OriginPro version 8.0J (Lightstone Inc, Tokyo, Japan), and R (The R Foundation for Statistical Computing, Perugia, Italy).<sup>30</sup> The probability of overall survival was calculated using the Kaplan-Meier method and compared using the log-rank test. The probabilities of transplant-related mortality, disease relapse, acute GVHD, chronic GVHD, and each cause of death were compared using the Grey test<sup>31</sup>

and analyzed using cumulative incidence analysis,<sup>30</sup> considering relapse, death without disease relapse, death without acute GVHD, death without chronic GVHD, and death without each cause as respective competing risks. The analysis was stratified for patients with standard-risk disease and high-risk disease to take into account the already recognized prognostic differences. The variables considered were recipient age at time of transplantation, sex, recipient cytomegalovirus serosta-

Table 1. Characteristics of the donors and recipients.

Variable	Standard-risk disease (n=93, 64%) Donor NKG2D haplotype				p	High-risk disease (n=52, 36%) Donor NKG2D haplotype				p
	HNK1 negative n=55, 59%		HNK1 negative n=38, 41%			HNK1 positive n=28, 54%		HNK1 negative n=24, 46%		
	N.	Ratio	N.	Ratio		N.	Ratio	N.	Ratio	
Age, years										
Recipient					0.08					0.39
Median	31		23			23		22		
Range	1-50		1-50			7-46		2-48		
Donor					0.54					
Median	33		28			34		29		0.02
Range	22-49		21-50			21-47		21-50		
Recipient NKG2D haplotype					0.17					0.48
HNK1 positive	33	60%	28	74%		19	68%	14	58%	
HNK1 negative	22	40%	10	26%		9	32%	10	42%	
Sex, male					0.37					0.77
Recipient	30	55%	23	61%		19	68%	15	63%	
Donor	42	76%	23	61%		19	68%	13	54%	
Recipient/donor sex					0.23					0.86
Sex matched	31	56%	20	53%		18	64%	16	67%	
Male/female	6	11%	9	24%		5	18%	5	21%	
Female/male	18	33%	9	24%		5	18%	3	13%	
Disease					0.86					0.99
Acute myeloid leukemia	14	25%	9	24%		14	50%	12	50%	
Acute lymphoblastic leukemia	10	18%	8	21%		10	36%	9	38%	
Myelodysplastic syndrome	6	11%	5	13%		0	0%	0	0%	
Malignant lymphoma	2	4%	3	8%		1	4%	1	4%	
Chronic myeloid leukemia	23	42%	13	34%		3	11%	2	8%	
ABO matching					0.37					0.18
Matched	35	64%	19	50%		14	50%	17	71%	
Major mismatch	11	20%	10	26%		6	21%	5	21%	
Minor mismatch	9	16%	9	24%		8	29%	2	8%	
Bi-directional	0	0%	1	3%		0	0%	1	4%	
Conditioning regimen					0.93					0.51
With total body irradiation	43	78%	30	79%		26	93%	21	88%	
Without total body irradiation	12	22%	8	21%		2	7%	3	13%	
Pretransplant CMV serostatus					0.30					0.99
CMV-negative recipient	14	25%	5	13%		6	27%	5	21%	
Missing data	4	7%	2	5%		5	18%	4	17%	
GVHD prophylaxis					0.58					0.11
With cyclosporine	51	93%	34	89%		27	96%	20	83%	
With tacrolimus	4	7%	4	11%		1	4%	4	17%	
TNC, ×10 <sup>7</sup> /kg					0.40					0.04
Median	5.4		5.8			5.8		8.2		
Range	2.3-14.6		2.3-57.6			2.9-20.0		2.4-42.8		
Engraftment	53	96%	38	100%	0.23	28	100%	23	96%	0.28

CMV: cytomegalovirus; TNC: total nucleated cell count harvested.