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*
Pre-transplantation factors			
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
Background disease			
AML	17	18	
ALL	12	12	
CML	6	3	
MDS	5	7	
Others	5	3	0.57
Donor/recipient CMV status			
Pos./Pos.	28	26	
Pos./Neg.	5	4	
Neg./Pos.	8	6	0.74
Conditioning regimen			
TBI	39	36	
Non-TBI	6	7	0.77
GVHD prophylaxis			
CYA–MTX	25	25	
TAC–MTX	16	16	0.59

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

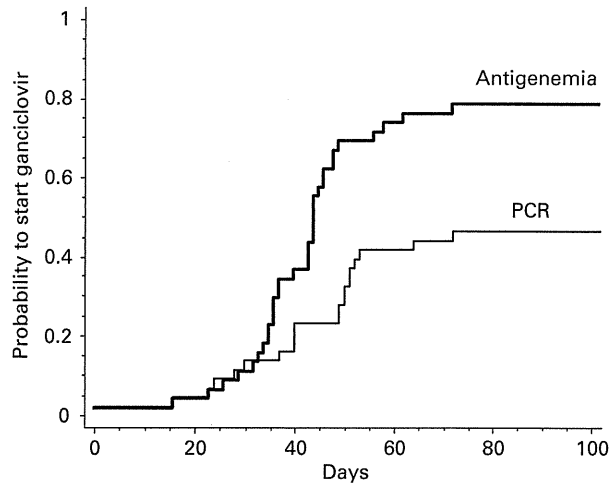

Figure 2 Days to start ganciclovir after transplantation.

Table 2 CMV-related events after engraftment

	Antigenemia (n=*45*)	*PCR* (n=*43*)	P-*value*
CMV reactivation[a]	40	27	0.0050
Start ganciclovir	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[b]	8	0	0.039
CMV disease			
Early (before day 100)	1	2	0.61
Late (after day 100)	0	1[c]	0.48

[a]Detection of antigenemia or DNA at any level.
[b]Increase in serum creatinine level by 0.5 mg per 100 ml or more from the baseline level.
[c]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.

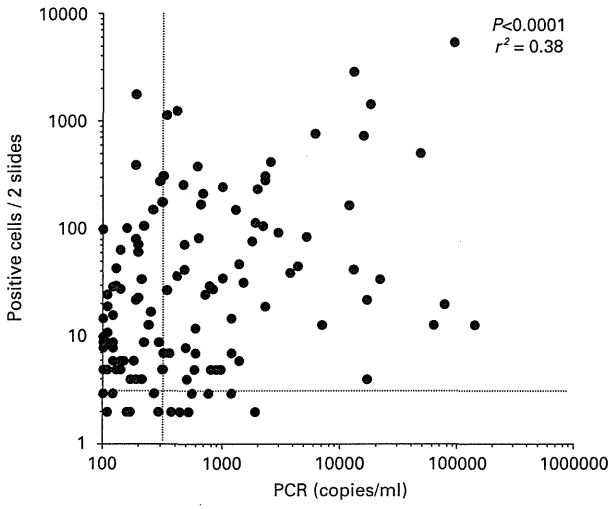

Figure 3 Correlation between the number of positive cells in the antigenemia assay and copy number by PCR.

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 ± 19.4 days and 140.8 ± 129.7 mg/kg in the antigenemia group and 20.8 ± 14.2 days and 118.4 ± 91.2 mg/kg in the PCR group ($P=0.64$ and $P=0.51$), respectively. Neutropenia with a neutrophil count of <500 per μl 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 ± 82.5 vs 126.9 ± 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 PCR group ($P=0.039$). The total dose of GCV was significantly higher in patients who developed renal impairment (255.0 ± 198.0 vs 106.0 ± 45.5, $P=0.0004$).

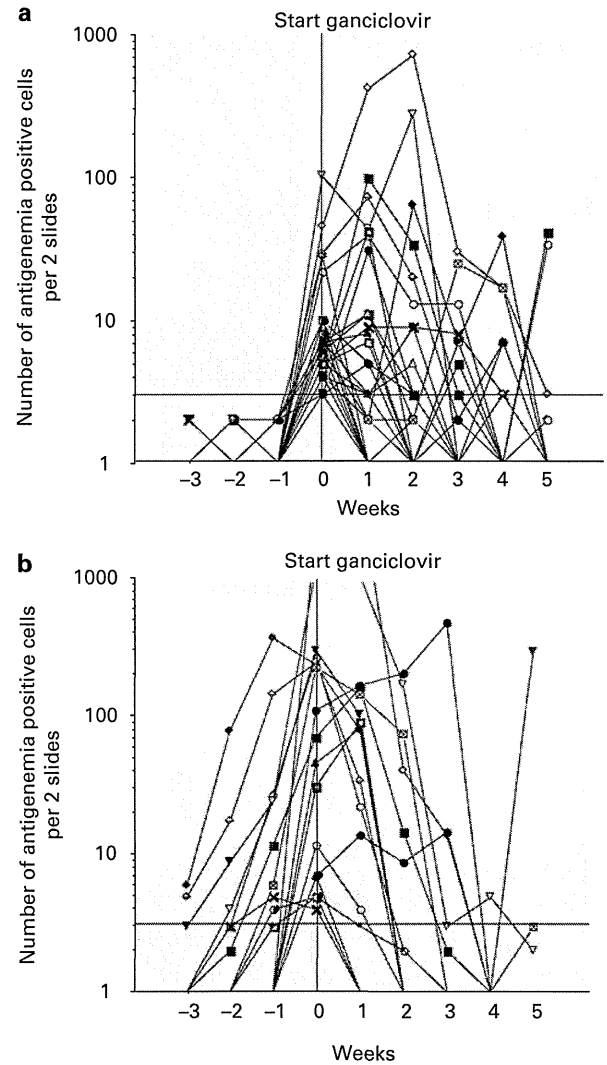

Figure 4 Serial changes in the number of antigenemia-positive cells in patients who received preemptive therapy in the antigenemia group (**a**) and in the PCR group (**b**). Week 0 represents the day ganciclovir was started.

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

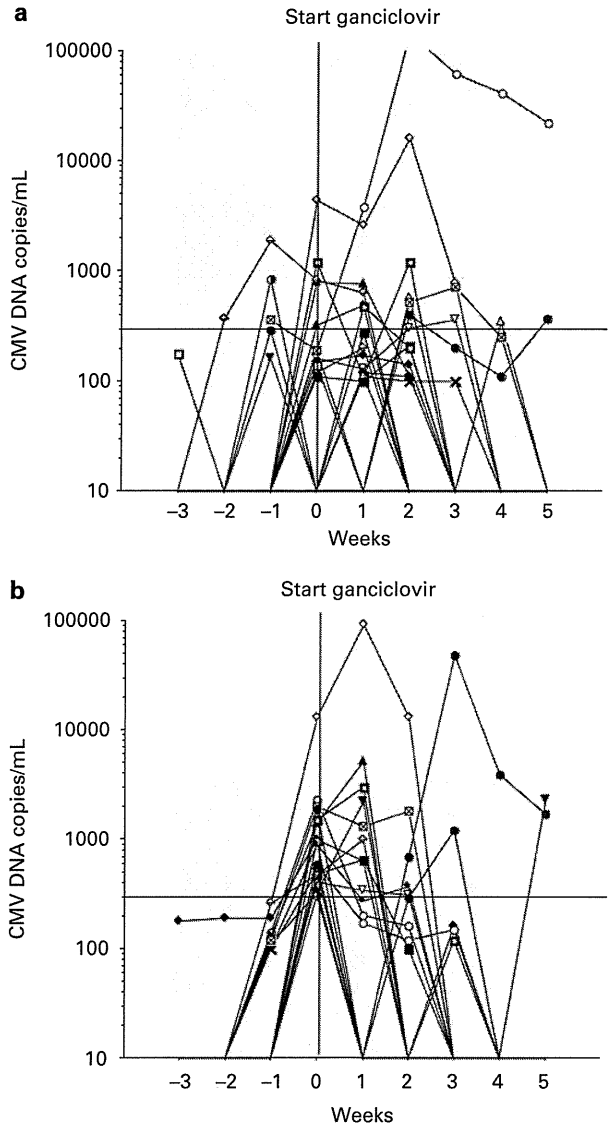

Figure 5 Serial changes in CMV DNA copy number in patients who received preemptive therapy in the antigenemia group (**a**) and in the PCR group (**b**). Week 0 represents the day ganciclovir was started.

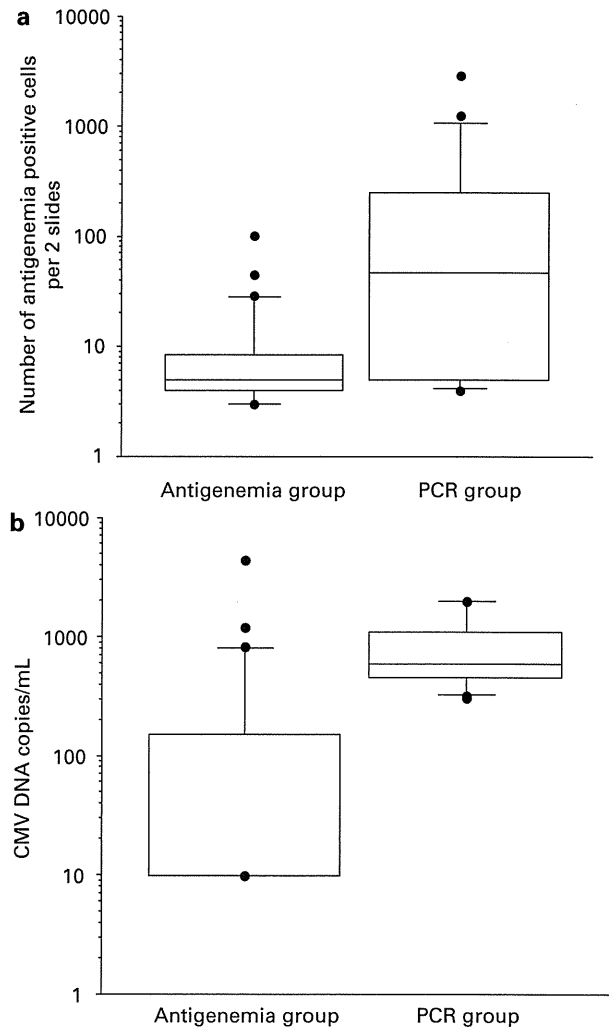

Figure 6 The number of antigenemia-positive cells (**a**) and the CMV DNA copy number at the start of preemptive therapy (**b**), grouped according to the randomization arm. The box-and-whisker plot shows 10, 25, 50, 75 and 90 percentile values. Outliers are indicated by dots.

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 even with a low threshold.[21] 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,[20] to transplantation from an unrelated donor.

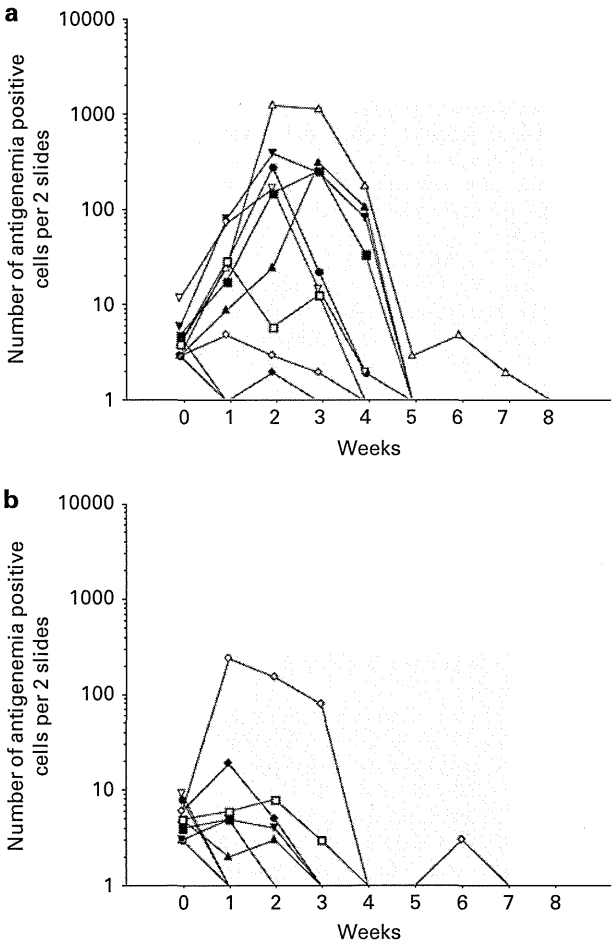

Figure 7 Serial changes in the number of antigenemia-positive cells in the PCR group patients who developed positive antigenemia that reached the threshold, but who did not receive ganciclovir. (**a**) Patients who developed grade II–IV acute GVHD or who received steroid at 0.5 mg/kg or more. (**b**)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

Although Boeckh *et al.*[3] 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.[9,22–24] 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.[18] 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,[6,7,20] 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.[25,26]

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.

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[a]	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[a]	99

[a]Preemptive therapy was started.

Conflict of interest

The authors declare no conflict of interest.

Acknowledgements

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References

1 Goodrich JM, Bowden RA, Fisher L, Keller C, Schoch G, Meyers JD. Ganciclovir prophylaxis to prevent cytomegalovirus disease after allogeneic marrow transplant. *Ann Intern Med* 1993; **118**: 173–178.
2 Winston DJ, Ho WG, Bartoni K, Du Mond C, Ebeling DF, Buhles WC *et al.* Ganciclovir prophylaxis of cytomegalovirus infection and disease in allogeneic bone marrow transplant recipients. Results of a placebo-controlled, double-blind trial. *Ann Intern Med* 1993; **118**: 179–184.
3 Boeckh M, Gooley TA, Myerson D, Cunningham T, Schoch G, Bowden RA. Cytomegalovirus pp65 antigenemia-guided early treatment with ganciclovir versus ganciclovir at engraftment after allogeneic marrow transplantation: a randomized double-blind study. *Blood* 1996; **88**: 4063–4071.
4 Boeckh M, Ljungman P. How we treat CMV in hematopoietic cell transplant recipients. *Blood* 2009; **113**: 5711–5719.
5 Ljungman P, Reusser P, de la Camara R, Einsele H, Engelhard D, Ribaud P *et al.* Management of CMV infections: recommendations from the infectious diseases working party of the EBMT. *Bone Marrow Transplant* 2004; **33**: 1075–1081.
6 Kanda Y, Mineishi S, Saito T, Seo S, Saito A, Suenaga K *et al.* Pre-emptive therapy against cytomegalovirus (CMV) disease guided by CMV antigenemia assay after allogeneic hematopoietic stem cell transplantation: a single-center experience in Japan. *Bone Marrow Transplant* 2001; **27**: 437–444.
7 Kanda Y, Mineishi S, Saito T, Saito A, Ohnishi M, Niiya H *et al.* Response-oriented preemptive therapy against cytomegalovirus disease with low-dose ganciclovir: a prospective evaluation. *Transplantation* 2002; **73**: 568–572.
8 Einsele H, Ehninger G, Hebart H, Wittkowski KM, Schuler U, Jahn G *et al.* Polymerase chain reaction monitoring reduces the incidence of cytomegalovirus disease and the duration and side effects of antiviral therapy after bone marrow transplantation. *Blood* 1995; **86**: 2815–2820.
9 Boeckh M, Gallez-Hawkins GM, Myerson D, Zaia JA, Bowden RA. Plasma polymerase chain reaction for cytomegalovirus DNA after allogeneic marrow transplantation: comparison with polymerase chain reaction using peripheral blood leukocytes, pp65 antigenemia, and viral culture. *Transplantation* 1997; **64**: 108–113.
10 Kanda Y, Chiba S, Suzuki T, Kami M, Yazaki Y, Hirai H. Time course analysis of semi-quantitative PCR and antigenaemia assay for prevention of cytomegalovirus disease after bone marrow transplantation. *Br J Haematol* 1998; **100**: 222–225.
11 Mori T, Okamoto S, Watanabe R, Yajima T, Iwao Y, Yamazaki R *et al.* Dose-adjusted preemptive therapy for cytomegalovirus disease based on real-time polymerase chain reaction after allogeneic hematopoietic stem cell transplantation. *Bone Marrow Transplant* 2002; **29**: 777–782.
12 Gerna G, Lilleri D, Caldera D, Furione M, Zenone Bragotti L, Alessandrino EP. Validation of a DNAemia cutoff for preemptive therapy of cytomegalovirus infection in adult hematopoietic stem cell transplant recipients. *Bone Marrow Transplant* 2008; **41**: 873–879.
13 Lilleri D, Gerna G, Furione M, Bernardo ME, Giorgiani G, Telli S *et al.* Use of a DNAemia cut-off for monitoring human cytomegalovirus infection reduces the number of preemptively treated children and young adults receiving hematopoietic stem-cell transplantation compared with qualitative pp65 antigenemia. *Blood* 2007; **110**: 2757–2760.
14 Verkruyse LA, Storch GA, Devine SM, Dipersio JF, Vij R. Once daily ganciclovir as initial pre-emptive therapy delayed until threshold CMV load > or = 10000 copies/ml: a safe and effective strategy for allogeneic stem cell transplant patients. *Bone Marrow Transplant* 2006; **37**: 51–56.
15 Nakai K, Kanda Y, Mineishi S, Saito T, Ohnishi M, Niiya H *et al.* Suspected delayed immune recovery against cytomegalovirus after reduced-intensity stem cell transplantation using anti-thymocyte globulin. *Bone Marrow Transplant* 2002; **29**: 237–241.
16 Kanda Y, Mineishi S, Nakai K, Saito T, Tanosaki R, Takaue Y. Frequent detection of rising cytomegalovirus antigenemia after allogeneic stem cell transplantation following a regimen containing antithymocyte globulin. *Blood* 2001; **97**: 3676–3677.
17 Kurihara T, Hayashi J, Ito A, Asai T. CMV antigenemia assay using indirect ALP-immunostaining in bone marrow transplant recipients. *Transplant Proc* 1996; **28**: 1750–1753.
18 Tanaka Y, Kanda Y, Kami M, Mori S, Hamaki T, Kusumi E *et al.* Monitoring cytomegalovirus infection by antigenemia assay and two distinct plasma real-time PCR methods after hematopoietic stem cell transplantation. *Bone Marrow Transplant* 2002; **30**: 315–319.
19 Asano-Mori Y, Kanda Y, Oshima K, Watanabe T, Shoda E, Motokura T *et al.* Pharmacokinetics of ganciclovir in haematopoietic stem cell transplantation recipients with or without renal impairment. *J Antimicrob Chemother* 2006; **57**: 1004–1007.
20 Asano-Mori Y, Kanda Y, Oshima K, Kako S, Shinohara A, Nakasone H *et al.* Clinical features of late cytomegalovirus infection after hematopoietic stem cell transplantation. *Int J Hematol* 2008; **87**: 310–318.
21 Mori T, Mori S, Kanda Y, Yakushiji K, Mineishi S, Takaue Y *et al.* Clinical significance of cytomegalovirus (CMV) antigenemia in the prediction and diagnosis of CMV gastrointestinal disease after allogeneic hematopoietic stem cell transplantation. *Bone Marrow Transplant* 2004; **33**: 431–434.
22 Boeckh M, Woogerd PM, Stevens-Ayers T, Ray CG, Bowden RA. Factors influencing detection of quantitative cytomegalovirus antigenemia. *J Clin Microbiol* 1994; **32**: 832–834.
23 Gerna G, Revello MG, Percivalle E, Morini F. Comparison of different immunostaining techniques and monoclonal antibodies to the lower matrix phosphoprotein (pp65) for optimal quantitation of human cytomegalovirus antigenemia. *J Clin Microbiol* 1992; **30**: 1232–1237.
24 Grundy JE, Ehrnst A, Einsele H, Emery VC, Hebart H, Prentice HG *et al.* A three-center European external quality control study of PCR for detection of cytomegalovirus DNA in blood. *J Clin Microbiol* 1996; **34**: 1166–1170.
25 Gerna G, Percivalle E, Torsellini M, Revello MG. Standardization of the human cytomegalovirus antigenemia assay by means of *in vitro*-generated pp65-positive peripheral blood polymorphonuclear leukocytes. *J Clin Microbiol* 1998; **36**: 3585–3589.
26 Verschuuren EA, Harmsen MC, Limburg PC, van Der Bij W, van Den Berg AP, Kas-Deelen AM *et al.* Towards standardization of the human cytomegalovirus antigenemia assay. *Intervirology* 1999; **42**: 382–389.

Mycophenolate and Tacrolimus for Graft-Versus-Host Disease Prophylaxis for Elderly After Cord Blood Transplantation: A Matched Pair Comparison With Tacrolimus Alone

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Background. The optimal graft-versus-host disease (GVHD) prophylaxis after umbilical cord blood transplantation has not been established. Our previous observation using single calcineurin inhibitors revealed a high incidence and severity of early immune-mediated complications, especially for older patients or those with poor performance status.
Methods. We conducted a single institute pilot study assessing the safety and effectiveness of mycophenolate mofetil (MMF) and tacrolimus (FK) combination as a GVHD prophylaxis for 29 patients (FK+MMF), and the results were compared with matched-pairs extracted from our historical database who received FK alone as GVHD prophylaxis (control).
Results. FK+MMF group showed superior engraftment rate compared with control group (cumulative incidence until day 60 posttransplant; 90%±0% vs. 69%±1%, $P=0.02$). A cumulative incidence of severe type preengraftment immune reactions was significantly decreased in FK+MMF group (16%±1%) compared with that of control group (52%±2%, $P=0.03$), and, remarkably, there was no nonrelapse mortality (NRM) observed up to day 30 posttransplant in FK+MMF group, whereas 21%±1% of NRM was observed in the control group. However, the incidences of acute and chronic GVHD, estimated overall and progression-free survivals were comparable between two groups.
Conclusions. MMF and FK in combination was well tolerated and decreased early NRM possibly by better control of preengraftment immune reactions. Subsequent NRM or disease progression needs to be overcome to further improve survival.

Keywords: Cord blood transplantation, GVHD prophylaxis, Mycophenolate mofetil, Tacrolimus, Elderly patients.

(*Transplantation* 2011;92: 366–371)

Although umbilical cord blood transplantation (UCBT) has been increasingly used as a curative treatment of hematological diseases, accompanying toxicity, especially early period posttransplant, has been a major problem (*1*, *2*). Our previous observation indicated that elderly patients were more vulnerable to early toxicity posttransplant, with nonrelapse mortality (NRM) being a major cause of treatment failure (*3*). Early immune-mediated complications, termed preengraftment immune reactions (PIR), were significant factors that negatively affected overall survival (OS) (*3–5*).

Various immunosuppressive drugs have been used for graft-versus-host disease (GVHD) prophylaxis in UCBT, including mycophenolate mofetil (MMF), (*6–8*) methotrexate (MTX), (*9–11*) corticosteroids, (*11*) anti-thymocyte globulin, (*12*, *13*), and sirolimus (*14*); mostly in combination with calcineurin inhibitors. So far, no available data indicate that one drug or combination is better than the other.

MMF is an inosine monophosphate dehydrogenase inhibitor that exerts its immunosuppressive effect by blocking the production of guanosine nucleotide synthesis through the

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de novo pathway (*15*). It has been extensively used in solid organ transplantations (*16*) and more recently, in hematopoietic cell transplantation (HCT) (*7, 17–19*). In HCT, less mucosal damage compared with MTX has been observed, (*19–21*) with a comparable incidence of GVHD, suggesting a potential advantage of MMF over MTX. It therefore seemed rational to incorporate MMF in reduced-intensity (RI) UCBT for patients at high risk for NRM. Since December 2005, MMF+tacrolimus (FK) combination was started to be used as GVHD prophylaxis in RI-UCBT as a pilot study for those who agreed to participate. The results were compared with that of those who performed RI-UCBT using FK alone extracted as matched pairs from our historical database.

RESULTS

Patients/Matched Controls

Table 1 shows the demography of the patient characteristics of two groups. A total of 89% of the control patients who had GVHD prophylaxis of FK alone were transplanted from 2004 to 2005, whereas 93% of the patients with FK+MMF were from 2006 to 2007 ($P<.0001$). The differences between the groups did not reach statistical significance in Eastern Cooperative Oncology Group (ECOG) performance status (PS), HCT-specific comorbidity index (HCT-CI) score, history of previous HCT, human leukocyte antigen (HLA) disparity to UCB, and conditioning regimen. The median FK concentrations (11.9±0.33 ng/mL in FK+MMF group vs. 12.6±0.47 ng/mL in control group, $P=0.46$) and the proportions of FK concentration more than or equal to 10 ng/mL during day 0 to the date of engraftment (72.4%±3.1% in FK+MMF group vs. 75.0%±4.0% in control group, $P=0.43$) were comparable in each group.

Engraftment

Twenty-seven patients in FK+MMF group achieved neutrophil engraftment, and all except 1 showed complete donor chimerism. The cumulative incidence of primary engraftment until day 60 posttransplant was 90%±0%, whereas that of control group was 69%±1% ($P=0.02$). Median time to engraftment was 19 days after transplantation both in FK+MMF group (range, 13–32 days) and control group (range, 12–33 days). Among the two patients in FK+MMF group who failed to engraft, one experienced disease recurrence before day 28, and the other experienced rejection of donor cells and was later found to have anti-HLA antibodies against one of the antigens expressed on donor cells. One patient in FK+MMF group who showed mixed chimerism on neutrophil engraftment, when 87.2% of total bone marrow (BM) cells were of donor origin, experienced early BM relapse of leukemia on day 30 posttransplant. There were three patients in control group who experienced hemophagocytic syndrome (HPS) early after transplant and resulted in early death before engraftment, whereas there was no such cases observed in FK+MMF group. Platelet recovery more than 20×10^9/L was observed in 17 patients, with a cumulative incidence of 59%±1% at day 100 posttransplant (median, 40 days; range, 25–70 days), whereas in control group, the cumulative incidence was 52%±1% (median, 40 days; range, 26–62 days, $P=0.69$).

TABLE 1. Patient, treatment, and donor umbilical cord blood characteristics

Characteristic	N (%) of patients FK+MMF	Control	P
Sex			0.38
Male	21 (72)	23 (79)	
Female	8 (28)	6 (21)	
Age (yr)			0.67
Median (range)	62 (52–70)	63 (56–69)	
Age distribution (yr)			
51–55	5 (17)	0	
56–60	4 (14)	9 (31)	
61–65	12 (41)	13 (45)	
66–70	8 (28)	7 (24)	
Diagnosis			0.11
AML/MDS	19 (66)	16 (55)	
ALL	2 (7)	5 (17)	
ML	5 (17)	5 (17)	
CML	0	3 (10)	
AA	3 (10)	0	
ECOG performance status			0.37
0	0	0	
1	22 (76)	17 (59)	
2	5 (17)	9 (31)	
3	2 (7)	3 (10)	
HCT-CI			0.25
0	9 (31)	18 (62)	
1	12 (41)	7 (24)	
2	1 (3)	1 (3)	
≥3	7 (24)	3 (10)	
Disease status			0.78
Standard risk	10 (34)	9 (31)	
High risk	19 (66)	20 (69)	
History of prior HCT			0.16
None	22 (76)	26 (90)	
Autologous	4 (14)	3 (10)	
Allogeneic	3 (10)	0	
Year of transplant			<0.0001
2004	0	11 (38)	
2005	2 (7)	12 (41)	
2006	7 (24)	6 (21)	
2007	20 (69)	4 (14)	
Conditioning regimen[a]			
Flu/Mel 140	8 (28)	1 (3)	
Flu/Mel 80-140/TBI 2-8	13 (45)	25 (86)	
Flu/Mel 80/Tespa 10	0	1 (3)	
Flu/Mel 80-140/Bu 8-16	4 (14)	0	
Flu/Bu 16	0	1 (3)	
Flu/Bu 8-16/TBI 2-4	3 (10)	1 (3)	
Flu/Bu 8/VP-16 450	1 (3)	0	
HLA disparity to UCB			0.22
0 antigen mismatch	1 (3)	1 (3)	
1 antigen mismatch	5 (17)	1 (3)	
2 antigen mismatch	23 (79)	27 (93)	
Total nucleated cell number			0.66
Median ($\times10^7$/kg)	2.4	2.31	
Range ($\times10^7$/kg)	2.0–4.5	1.91–4.76	
$CD34^+$ cell number			0.15
Median ($\times10^5$/kg)	0.9	0.81	
Range ($\times10^5$/kg)	0.11–2.32	0.11–1.9	

[a] Units for each number are as follows: Mel (mg/m^2), TBI (Gy), Tespa (mg/kg), Bu doses: oral (1 dose=1 mg/kg) or iv (1 dose=0.8 mg/kg), and VP-16 (mg/m^2).

AML, acute myeloid leukemia; MDS, myelodysplastic syndrome; ALL, acute lymphoblastic leukemia; ML, malignant lymphoma; CML, chronic myeloid leukemia; AA, aplastic anemia; HCT-CI, hematopoietic cell transplantation-specific comorbidity index; Flu, fludarabine; Mel, melphalan; TBI, total body irradiation; Bu, busulfan; VP-16, etoposide; and UCB, umbilical cord blood; FK, tacrolimus; MMF, mycophenolate mofetil; ECOG, Eastern Cooperative Oncology Group; HLA, human leukocyte antigen.

TABLE 2. Incidence of PIR and GVHD

	FK+MMF (N)	Control (N)
PIR (n=29)		
No. of evaluable[a]	29	28
Yes	22	23
Severe type	4	10
Acute GVHD		
No. of evaluable[b]	27	20
Grade I	4	4
Grade II	7	2
Grade III	7	5
Grade IV	4	3
Chronic GVHD		
No. of evaluable[c]	13	11
Limited	1	2
Extensive	1	2

[a] Those who showed clinical symptoms characteristic to PIR, and those who survived longer than 27 d posttransplant without PIR.

[b] Those who engrafted without disease progression.

[c] Those who survived beyond day 100 posttransplant without disease progression.

PIR, preengraftment immune reactions; GVHD, graft-versus-host disease; FK, tacrolimus; MMF, mycophenolate mofetil.

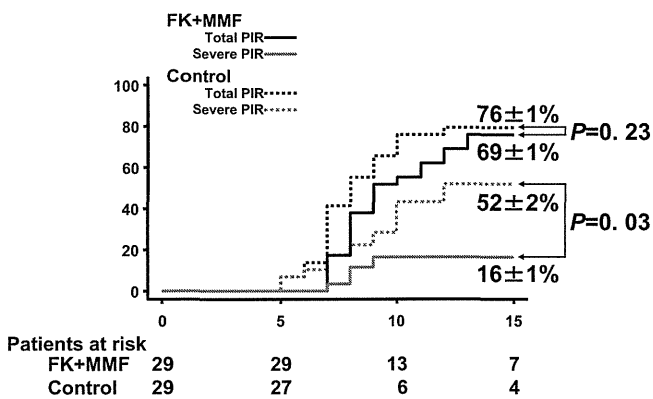

FIGURE 1. Cumulative incidences of preengraftment immune reactions (PIR) after RI-UCBT according to tacrolimus (FK)+mycophenolate mofetil (MMF) or FK alone graft-versus-host disease (GVHD) prophylaxis. The overall incidences of PIR in FK+MMF group (*black solid line*), in control group (*black dotted line*), and the incidences of severe type of PIR in FK+MMF group (*gray solid line*), and in control group (*gray dotted line*) were plotted. There was significant reduction of severe type of PIR in FK+MMF group compared with that in control group (P=0.03).

PIR and GVHD

In FK+MMF group, 22 of 29 patients experienced clinical symptoms defined as PIR, whereas in control group, 23 of 28 evaluable patients did (Table 2). Cumulative incidences of PIR in both groups were comparable each other (76%±1% in control group and 69%±1% in FK+MMF group, P=0.23, Fig. 1) and were similar to that reported in our previous publication (*3*). However, the cumulative incidence of severe type of PIR, defined by the criteria described in materials and methods section, in the FK+MMF group was lower (16%±1%) than that of control group (52%±2%) with statistical significance (P=0.03, Fig. 1).

TABLE 3. Causes of death

	FK+MMF, N (%)	Control, N (%)
NRM	9 (45)	11 (65)
GVHD	5 (25)	3 (18)
IPS	4 (20)	1 (6)
Infection	0	5 (29)
CNS complication	0	2 (12)
Relapse/disease progression	11 (55)	6 (35)
Total	20	17

FK, tacrolimus; MMF, mycophenolate mofetil; NRM, nonrelapse mortality; GVHD, graft-versus-host disease; IPS, idiopathic pneumonia syndrome; CNS, central nervous system.

In FK+MMF group, 22 of 27 evaluable patients developed acute GVHD, and 18 of them were grade II and higher. In control group, 14 of 20 evaluable patients had acute GVHD, and 10 of them were grade II and higher (Table 2). Cumulative incidences of grade II and higher acute GVHD at day 100 posttransplant were 63%±1% in FK+MMF and 35%±1% in control group (P=0.09). Chronic GVHD was observed in two of 13 FK+MMF group and four of 11 control group patients who survived longer than 100 days posttransplant without disease progression (Table 2). Cumulative incidences of chronic GVHD at 2 years posttransplant were 7%±0% in FK+MMF and 16%±1% in control group (P=0.35).

Survival, Disease Progression, and NRM

At the time of analysis, 9 FK+MMF group patients survived for a median of 980 days (range, 145–1430 days) after transplantation, whereas 12 control group patients were alive for a median of 1073 days (range, 49–2071 days). The Kaplan-Meier estimates of OS and progression-free survival (PFS) at 2 year posttransplant in FK+MMF group were 33%±9% and 21%±8%, whereas those in control group were 45%±10% and 34%±9%, respectively. The differences were not statistically significant (P=0.83 for OS, and P=0.75 for PFS).

Thirteen patients in FK+MMF group showed progression of the underlying disease at a median of 84 days (range, 19–344 days) after transplantation, and 11 of these patients died of the disease (Table 3). In control group, 9 patients did so at a median of 126 days (range, 12–1084 days) and 6 died of the disease. The cumulative incidences of disease progression at 2 years were 46%±1% in FK+MMF group and 29%±1% in control group, respectively (P=0.29).

Nine in FK+MMF group died of nonrelapse causes, whereas in control group patients, 11 NRM were observed (Table 3). GVHD and noninfectious pulmonary complications were observed in both groups as cause of death. None of the FK+MMF group died from infections as a sole reason of death, whereas five of the control group did. There was no death before day 30 posttransplant in FK+MMF group, whereas six in control group did. The cumulative incidences of NRM at day 30, 100, 365 were 0%±0%, 21%±1%, 28%±1% in FK+MMF group, and 21%±1%, 35%±1%,

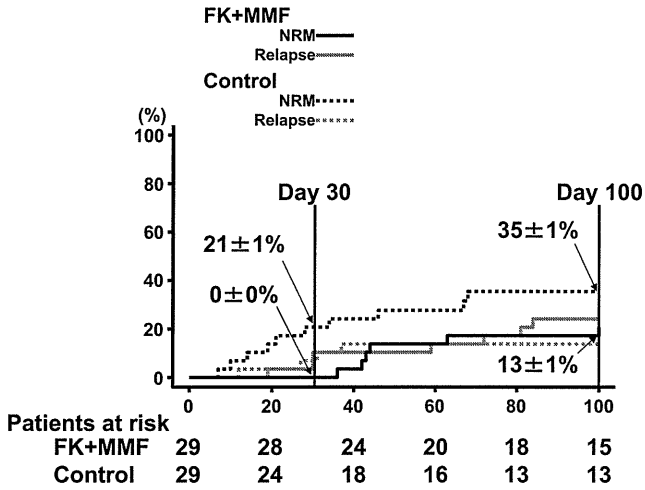

FIGURE 2. Day 100 nonrelapse mortality (NRM) and disease progression. Cumulative incidence estimates of NRM (*black line*) and disease progression (*gray line*) up to day 100 posttransplant for tacrolimus (FK)+mycophenolate mofetil (MMF) group (*solid line*) and control group (*dotted line*) were plotted. There were no NRM within 30 days posttransplant in FK+MMF group, whereas 21%±1% NRM were estimated in control group (P=0.01).

39%±1% in control group, respectively (P=0.01, P=0.17, P=0.29, Fig. 2).

DISCUSSION

The most remarkable observation in this study was that higher rate of neutrophil recovery and no early deaths before day 30 posttransplant were observed in FK+MMF group despite the patients' poor conditions before transplant, that is, all were older than 50 years and 69% of them had some comorbidities. Although the incidence of PIR in FK+MMF group was comparable with control group, the severity of PIR was less and thus did not result in severe organ damage early after transplant. There was no death directly caused by infections in FK+MMF group. We have reported higher incidence of HPS after RI-UCBT, which has been reasoned to be the delayed engraftment or graft failure (*22*). Interestingly, majority of the suffered BM cells were donor cell dominant, indicating HPS was mediated by donor-derived immune cells. Moreover, we have reported HLA mismatch in GVH direction, not in host-versus-graft direction, affected negatively to successful engraftment (*23*). All these facts fit well to the idea that hyperimmune reactions caused by donor cord blood (CB) cells may play crucial role in high rate of early NRM. Because there were no case of HPS in FK+MMF group, MMF may have promoted engraftment by sufficiently suppressing immune reactions of CB cells and preventing development of hemophagocytosis, which may also have reduced the incidence of severe infections. The presence of this type of hyperimmune reactions after UCBT has recently been recognized by others (*24*). The differences in incidence of PIR may have been affected by agents included in pretransplant conditioning, such as antithymocyte globulin, or by GVHD prophylaxis including corticosteroids or intravenous MMF.

Despite the present observation that the combination of MMF and FK succeeded in reducing early NRM, the incidence and severity of GVHD was not altered. Because most of the patients in the present study had advanced disease status, MMF was discontinued or started to be tapered on the day of neutrophil engraftment, which may have been responsible for this results. Much longer administration of MMF has been used in the setting of matched unrelated BM/peripheral blood (PB) transplantation (*7*). In addition, MMF was administered at 15 mg/kg twice daily in this study, which is the common dosing schedule in the settings of solid organ transplant (*16*). Several recent reports from Minnesota and Seattle considered 15 mg/kg three times daily as more appropriate based on pharmacokinetic data obtained from HCT recipients (*7*, *17*, *25*). A serum concentration measurement of mycophenolic acid, which was not assessed in this study, is needed to determine the optimal dosing of MMF.

Although NRM early after UCBT was significantly reduced in FK+MMF group, OS and PFS at 2 year posttransplant were still comparable with those of control group. Fifty-five percent of the deaths were from disease relapse or progression. Although MMF may have a beneficial effect on early survival after transplant by reducing severe immune reactions, it may increase the risk of disease progression for those who have active disease with a high risk of disease recurrence. According to previous publications, relapse rate is comparable in CB and unrelated BM/PB recipients despite lower incidences of chronic GVHD in CB recipients (*26*, *27*), early immune reactions may have impact on reducing disease relapse. Because this is a relatively small sized, retrospective study, the presence of uncontrolled bias cannot be excluded. Prospectively conducted larger studies are warranted to further confirm the results.

In conclusion, MMF, used in combination with FK as GHVD prophylaxis in elderly patients with advanced hematologic diseases with or without comorbidities, may reduce early mortality posttransplant by regulating severe PIR and thus protecting patients from severe organ damage or HPS. An optimal dosing schedule of MMF needs to be determined prospectively using more homogenous populations.

MATERIALS AND METHODS

Patients

The initial pilot study included patients aged 51 years and older who underwent RI-UCBT using MMF+FK combination as GVHD prophylaxis at our institute from December 2005 through December 2007. Patients were eligible for this study if they had any hematologic malignancies at high risk for relapse or severe aplastic anemia refractory to standard immunosuppressive therapy and were unable to find suitable related or unrelated BM/PB donors within reasonable periods relative to their disease conditions. Patients with acute leukemia could be at first remission but at high risk for relapse due to adverse cytogenetic abnormalities, have a previous hematologic disorder, or be at any status beyond first remission. Patients with myelodysplastic syndrome (MDS) had to be refractory anemia (RA) with excess of blasts or chronic myelomonocytic leukemia, or have RA with transfusion dependency or severe neutropenia. Malignant lymphoma (ML) patients had to be beyond first remission. Patients who had end-stage cardiac dysfunction (left ventricular ejection fraction <35%), pulmonary dysfunction (SpO_2 <90% in room air), or active serious infection at the time of transplantation were not eligible. All patients gave written informed consent. Twenty-nine patients were enrolled and subjected to the matched pair analysis as below.

Selection of Matched Controls and Matching Variables

A matched-pair control group (GVHD prophylaxis with FK alone) for 29 patients who used MMF+FK combination was obtained by selecting one of the most recently transplanted control patients from our historical RICBT database from 2004 to 2007 after excluding those who met exclusion criteria of the pilot study described earlier. Controls were individually matched to cases on a 1:1 ratio. Matching was attempted for the following criteria applied in the order listed: age at transplantation (51–60, 61–70 years), disease risk (standard risk vs. high risk, acute leukemia, chronic myeloid leukemia, or ML in complete remission, MDS RA, aplastic anemia patients were categorized as standard risk, and all the others were as high risk), ECOG PS (PS 0–1, 2–3), pretransplant conditioning (busulfan containing vs. others), number of serological HLA mismatch (0–1, 2), HCT-CI (0–1, ≥2), total nucleated cell dose infused (≤2.3, >2.3×10^7/kg), and $CD34^+$ cell dose infused (≤0.8, >0.8×10^5/kg). To avoid any potential selection bias, matching was blinded, and only the patient's initials and pretreatment variables were known. This retrospective analysis was approved by the institutional review board.

One hundred percent matching was achieved for age group; 97% for disease risk (high risk, 66% of FK+MMF patients vs. 69% of control patients; $P=0.78$); 83% for ECOG PS (≥2 score, 32% of FK+MMF patients vs. 41% of FK alone patients; $P=0.16$); 72% for HCT-CI (≥2 score, 28% of FK+MMF patients vs. 14% of control patients; $P=0.19$); and number of serological HLA mismatch (2 antigens, 79% of FK+MMF patients vs. 93% of control patients; $P=0.13$); 86% for pretransplant conditioning (inclusion of busulfan, 24% of FK+MMF patients vs. 7% of control patients; $P=0.07$); 69% for total nucleated cell dose (≤2.3×10^7/kg, 41% of FK+MMF patients vs. 45% of control patients; $P=0.79$); and 62% for $CD34^+$ cell dose (≤0.8×10^5/kg, 45% of FK+MMF patients vs. 48% of control patients; $P=0.79$). Characteristics of the studied patients in both groups were shown in Table 1. Patients' comorbidity was assessed by a previously reported scoring system (*28*).

Donor Selection

UCB units were obtained from the Japanese Cord Blood Bank Network. HLA-A, -B, and -DR antigens were identified by serologic typing. UCB grafts had at least four of six HLA-A, -B, and -DR antigens that were matched to the recipient and had a cryopreserved cell dose of at least 1.9×10^7 nucleated cells per kg of recipient body weight.

Conditioning Regimens and Postgrafting Immunosuppression

Pretransplant conditionings were primarily RI regimens including 125 to 180 mg/m^2 of fludarabine (25 mg/m^2 for 5 days or 30 mg/m^2 for 6 days). Antithymocyte globulin was not incorporated. Granulocyte colony-stimulating factor (G-CSF) was started on day 1 posttransplant. Detailed information is shown in Table 1. Immunosuppressive therapy with FK (0.03 mg/kg continuous infusion, aiming for 12 to 17 ng/mL by at least three times a week measurement) with or without MMF (15 mg/kg twice daily) were started on day −1. MMF was discontinued or started to taper down on the day of neutrophil engraftment in the absence of active GVHD.

Definition of Engraftment, Preengraftment Immune Reactions, and Endpoints

Engraftment was defined as absolute neutrophil count more than 0.5×10^9/L for 3 consecutive days. Chimerism was assessed using fluorescent in situ hybridization in sex-mismatched donor-recipient pairs, or polymerase chain reaction for a variable number of tandem repeats with donor cells detected at a sensitivity of 10% in sex-matched pairs. Whole blood or BM cells were assessed at the time of granulocyte engraftment. Complete donor-type chimerism was defined when donor cells consisted of more than 90% of analyzed cells. PIR was characterized by the presence of at least three of the following symptoms with no direct consequences of infection or adverse effects of medication six or more days before engraftment, as described previously (*4*, *5*): a high fever (>38.5°C), skin eruptions, body weight gain greater than 5% of baseline, or peripheral edema. Those who had all four symptoms and at least two of the following criteria indicating severe organ damage were classified as severe type; (1) SpO_2 less than 92% or pleural/pericardial effusions present; (2) serum creatinine level more than or equal to 3 times of baseline; (3) total bilirubin level more than 3 mg/dL or asparatate aminotransferase/alanine aminotransferase levels more than three times of upper limit of normal; and (4) development of hemophagocytosis in BM.

The main parameters analyzed between groups were as follows: (1) cumulative incidences of neutrophil or platelet engraftment; (2) cumulative incidences of NRM and relapse; (3) incidences of PIR, acute and chronic GVHD; and (4) overall and progression-free survival (OS and PFS). The analysis was performed as of April, 2010. OS was calculated from the day of transplantation until death from any cause or last follow-up. PFS was calculated from the day of transplantation until relapse, second transplantation due to engraftment failure, or death from any cause or last follow-up. NRM was defined as death in the absence of disease progression. Deaths occurring after disease progression were categorized as relapse regardless of the cause of death. Infection was considered the cause of death when bacterial, viral, or fungal infection was determined to be the proximate cause of death in patients who had not relapsed. Patients underwent BM aspiration at the time of engraftment or if clinically indicated. Relapse for acute myeloid leukemia, acute lymphoblastic leukemia, MDS, or chronic myeloid leukemia was determined by flow cytometric, morphologic, or cytogenetic evidence of malignant or dysplastic cells with clonal markers similar to those observed before transplantation. Relapse for ML was defined as progressive adenopathy or BM involvement. Acute and chronic GVHD were defined and graded by standard criteria (*29*). Relapse and NRM rates were estimated using cumulative incidence analysis and were considered competing risks (*30*). Similarly, in the analysis of PIR rates, death due to other causes or relapse leading to early withdrawal of immune suppression were considered competing risks.

Statistical Methods

Chi-square test was used to compare patient characteristics between two groups in matched-pair analysis. For continuous variables, Mann-Whitney nonparametric test was used. The probabilities of OS and PFS were estimated and plotted using the Kaplan-Meier method (*31*). Cumulative incidence curves were drawn using Gray's method (*32*). The level of significance in all cases was set at P less than 0.05. The effect of various categoric variables on survival probabilities was studied with the log-rank test. A Cox proportional hazard model with limited variables because of small sample was used to determine the significance of multiple variables in determining these outcomes. All analyses were carried out using StatView statistical software for Kaplan-Meier curve, and S-PLUS software (Mathsoft, Seattle, WA) for cumulative incidence curve.

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REFERENCES

1. Brunstein CG, Wagner JE. Cord blood transplantation for adults. *Vox Sang* 2006; 91: 195.
2. Schoemans H, Theunissen K, Maertens J, et al. Adult umbilical cord blood transplantation: A comprehensive review. *Bone Marrow Transplant* 2006; 38: 83.
3. Uchida N, Wake A, Takagi S, et al. Umbilical cord blood transplantation after reduced-intensity conditioning for elderly patients with hematologic diseases. *Biol Blood Marrow Transplant* 2008; 14: 583.
4. Miyakoshi S, Yuji K, Kami M, et al. Successful engraftment after reduced-intensity umbilical cord blood transplantation for adult patients with advanced hematological diseases. *Clin Cancer Res* 2004; 10: 3586.
5. Kishi Y, Kami M, Miyakoshi S, et al. Early immune reaction after reduced-intensity cord-blood transplantation for adult patients. *Transplantation* 2005; 80: 34.
6. Brunstein CG, Barker JN, Weisdorf DJ, et al. Umbilical cord blood transplantation after nonmyeloablative conditioning: Impact on trans-

plantation outcomes in 110 adults with hematologic disease. *Blood* 2007; 110: 3064.
7. Giaccone L, McCune JS, Maris MB, et al. Pharmacodynamics of mycophenolate mofetil after nonmyeloablative conditioning and unrelated donor hematopoietic cell transplantation. *Blood* 2005; 106: 4381.
8. Osunkwo I, Bessmertny O, Harrison L, et al. A pilot study of tacrolimus and mycophenolate mofetil graft-versus-host disease prophylaxis in childhood and adolescent allogeneic stem cell transplant recipients. *Biol Blood Marrow Transplant* 2004; 10: 246.
9. Takahashi S, Ooi J, Tomonari A, et al. Comparative single-institute analysis of cord blood transplantation from unrelated donors with bone marrow or peripheral blood stem-cell transplants from related donors in adult patients with hematologic malignancies after myeloablative conditioning regimen. *Blood* 2007; 109: 1322.
10. Narimatsu H, Terakura S, Matsuo K, et al. Short-term methotrexate could reduce early immune reactions and improve outcomes in umbilical cord blood transplantation for adults. *Bone Marrow Transplant* 2007; 39: 31.
11. Wagner JE, Barker JN, DeFor TE, et al. Transplantation of unrelated donor umbilical cord blood in 102 patients with malignant and nonmalignant diseases: Influence of CD34 cell dose and HLA disparity on treatment-related mortality and survival. *Blood* 2002; 100: 1611.
12. Sanz GF, Saavedra S, Planelles D, et al. Standardized, unrelated donor cord blood transplantation in adults with hematologic malignancies. *Blood* 2001; 98: 2332.
13. Lekakis L, Giralt S, Couriel D, et al. Phase II study of unrelated cord blood transplantation for adults with high-risk hematologic malignancies. *Bone Marrow Transplant* 2006; 38: 421.
14. Culter CM, Stevenson K, Kim HT, et al. Double umbilical cord blood transplantation with reduced intensity conditioning and sirolimus-based GVHD prophylaxis. *Bone Marrow Transplant* 2011; 46: 659.
15. Lipsky JJ. Mycophenolate mofetil. *Lancet* 1996; 348: 1357.
16. Bardsley-Elliot A, Noble S, Foster RH. Mycophenolate mofetil: A review of its use in the management of solid organ transplantation. *BioDrugs* 1999; 12: 363.
17. Jacobson P, Rogosheske J, Barker JN, et al. Relationship of mycophenolic acid exposure to clinical outcome after hematopoietic cell transplantation. *Clin Pharmacol Ther* 2005; 78: 486.
18. Ballen KK, Spitzer TR, Yeap BY, et al. Double unrelated reduced-intensity umbilical cord blood transplantation in adults. *Biol Blood Marrow Transplant* 2007; 13: 82.
19. Bolwell B, Sobecks R, Pohlman B, et al. A prospective randomized trial comparing cyclosporine and short course methotrexate with cyclosporine and mycophenolate mofetil for GVHD prophylaxis in myeloablative allogeneic bone marrow transplantation. *Bone Marrow Transplant* 2004; 34: 621.
20. Cutler C, Li S, Kim HT, et al. Mucositis after allogeneic hematopoietic stem cell transplantation: A cohort study of methotrexate- and non-methotrexate-containing graft-versus-host disease prophylaxis regimens. *Biol Blood Marrow Transplant* 2005; 11: 383.
21. Pohlreich D, Vitek A, Maalouf J, et al. Decreased risk of acute gastrointestinal toxicity when substituting methotrexate with mycophenolate mofetil in the prevention of graft-versus-host disease in stem cell transplantation following myeloablative conditioning regimens. *Bone Marrow Transplant* 2006; 37: 235.
22. Takagi S, Masuoka K, Uchida N, et al. High incidence of haemophagocytic syndrome following umbilical cord blood transplantation for adults. *Br J Haematol* 2009; 147: 543.
23. Matsuno N, Wake A, Uchida N, et al. Impact of HLA disparity in the graft-versus-host direction on engraftment in adult patients receiving reduced-intensity cord blood transplantation. *Blood* 2009; 114: 1689.
24. Patel KJ, Rice RD, Hawke R, et al. Pre-engraftment syndrome after double-unit cord blood transplantation: A distinct syndrome not associated with acute graft-versus-host disease. *Biol Blood Marrow Transplant* 2010; 16: 435.
25. Nash RA, Johnston L, Parker P, et al. A phase I/II study of mycophenolate mofetil in combination with cyclosporine for prophylaxis of acute graft-versus-host disease after myeloablative conditioning and allogeneic hematopoietic cell transplantation. *Biol Blood Marrow Transplant* 2005; 11: 495.
26. Barker JN, Davies SM, DeFor T, et al. Survival after transplantation of unrelated donor umbilical cord blood is comparable to that of human leukocyte antigen-matched unrelated donor bone marrow: Results of a matched-pair analysis. *Blood* 2001; 97: 2957.
27. Laughlin MJ, Eapen M, Rubinstein P, et al. Outcomes after transplantation of cord blood or bone marrow from unrelated donors in adults with leukemia. *N Engl J Med* 2004; 351: 2265.
28. Sorror ML, Giralt S, Sandmaier BM, et al. Hematopoietic cell transplantation specific comorbidity index as an outcome predictor for patients with acute myeloid leukemia in first remission: Combined FHCRC and MDACC experiences. *Blood* 2007; 110: 4606.
29. Sullivan KM. Graft-versus-host-disease [ed. 4]. Cambridge, MA: Blackwell Science 1999.
30. Gooley TA, Leisenring W, Crowley J, et al. Estimation of failure probabilities in the presence of competing risks: New representations of old estimators. *Stat Med* 1999; 18: 695.
31. Kaplan EL, Meier P. Nonparametric estimation from incomplete observations. *J Am Stat Assoc* 1958; 53: 457.
32. Gray RJ. A class of k-sample tests for comparing the cumulative incidence of a competing risk. *Ann Statistics* 1988; 116: 1141.

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LETTER TO THE EDITOR

Unexpectedly high AUC levels in a child who received intravenous busulfan before stem cell transplantation

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A 6-year-old boy underwent a craniotomy with subtotal resection of the cerebellar vermian tumor. Microscopic examination showed medulloblastoma. Brain magnetic resonance imaging after resection did not show residual disease. He subsequently received four cycles of ICE (ifosfamide, cisplatin, and etoposide) chemotherapy followed by whole craniospinal (24 Gy) and local (30 Gy) radiation therapy. He eventually received tandem high-dose chemotherapy. Conditioning regimens for the first and the second were carboplatin (1200 mg/m^2) + thiotepa (750 mg/m^2) and busulfan 1.1 mg/kg/dose intravenous every 6 h for total 16 doses (17.6 mg/kg) + melphalan 70 mg/m^2 once daily intravenous for 2 days (140 mg/m^2), respectively. Autologous PBSC were infused after high-dose chemotherapy. The numbers of CD-34-positive cells were 5.2×10^6/kg in the first and 7.0×10^6/kg in the second, respectively. Engraftment after the second autologous PBSCT was achieved on day 11. Brain magnetic resonance imaging on day 25 of the second autologous PBSCT showed no evidence of recurrence.

On day 64 of the second autologous PBSCT, he developed sudden dyspnea with 90% oxygen saturation in room air. A chest X-ray film showed a ground-glass appearance in both lungs. Chest computed tomography scan also showed bilateral areas of ground-glass opacities, and no centrilobular micronodule. Aspergillus, candida, and cryptococcal antigen for the serum were negative. The β-D-glucan level was significantly elevated (234.4 pg/ml). Intravenous cotrimoxazole therapy was initiated, because pneumocystis pneumonia was thought to have caused pneumonia based on the clinical course. He was placed on mechanical ventilation and methylprednisolone pulse therapy (30 mg/kg × 3 days) was started on day 72. His respiratory condition improved quickly with these treatments and mechanical ventilation was discontinued on day 77. Although polymerase chain reaction for *Pneumocystis jirovecii* in bronchoalveolar lavage fluid was negative, we diagnosed the cause of his pulmonary disorder had been pneumocystis pneumonia. His respiratory condition deteriorated again on day 82. Despite the second course of methylprednisolone pulse and continued cotrimoxazole therapy, he was placed on mechanical ventilation again on day 85 (Figure 1a). No pathogenic bacterial, fungal, or viral agents, including cytomegalovirus were identified by microbiological cultures and serological studies. The β-D-glucan level was also decreased to 33.4 pg/ml. Although various antibiotics, ganciclovir, antifungal drugs, and cotrimoxazole therapy were continued, his pulmonary oxygenation became worse day-by-day. He died on day 133 of the second autologous PBSCT because of respiratory failure (Figure 1b).

An autopsy was performed with the consent of his parents. Microscopically, organizing diffuse alveolar damage was seen, and nuclear enlargement, hyperchromasia, and pleomorphism were seen along alveolar and bronchial epithelium. This cytologic atypia was seen not only in the lung, but also in the urothelium of the renal pelvis. There was no evidence of bacterial, fungal, or viral infection. These pathological findings were consistent with busulfan-induced lung disease. Organizing diffuse alveolar damage is the most common manifestation of busulfan lung toxicity and is associated with bronchiolar and alveolar epithelium atypia.[1] This cytologic atypia is often seen extrapulmonary sites, including urinary bladder, breast, and uterine cervix.[1] The incidence of pulmonary toxicity after high-dose oral busulfan therapy before stem cell transplantation has been reported to be 3.6%.[2] Corticosteroids are effective for treating this disease to various degrees. Although some patients improve, others progress and die.[1]

It has become evident posthumously that busulfan areas under the drug plasma concentration–time curve (AUC) levels at the 1st and 9th doses were significantly elevated (2353 μM × min, 2347 μM × min) (target AUC; 900–1500 μM × min). Busulfan concentrations for the 1st and 9th doses and the accumulation of intravenous busulfan in plasma were assayed using a high-performance liquid chromatography system (Figure 2).[3] Plasma concentrations were analyzed by the non-compartmental method using WinNonlin (version 5.2.1; Pharsight Corp., Mountain View, CA, USA). The AUC from time 0 to infinity (AUC_{inf}) for the 1st dose and at steady state (AUC_{ss}) for the 9th dose was calculated using linear trapezoidal rule. The relationship between high busulfan AUC levels and the occurrence of busulfan-induced lung disease has not been established,[4] although high busulfan AUC levels are commonly associated with hepatic veno-occlusive disease.[5]

The cause of high busulfan AUC levels in our patient is still unclear. He did not have the distinct liver or renal disorder at the time of busulfan administration. Busulfan is metabolized in the liver through conjugation with glutathione by glutathione *S*-transferase (GST) enzymes.[6] GSTA1 is the predominant isoform of GST, which catalyzes the conjugation of busulfan with glutathione. Polymorphisms in GSTA1 are thought to be associated with alterations in the pharmacokinetics of busulfan.[7]

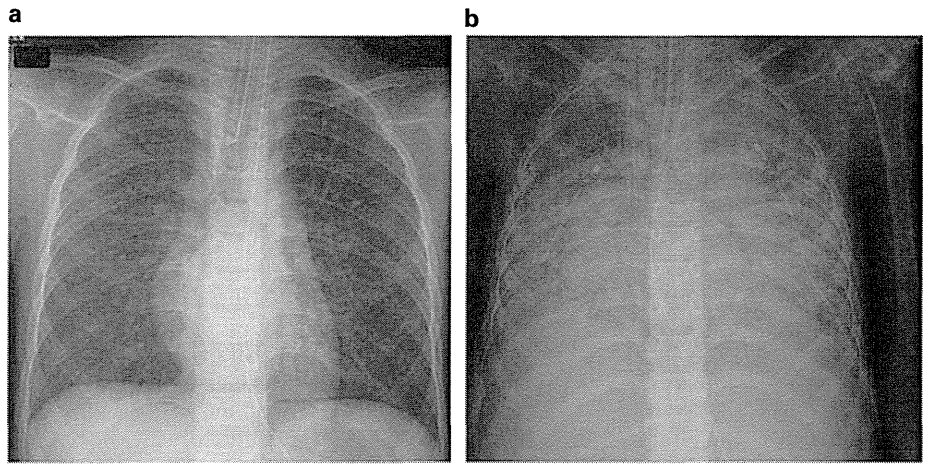

Figure 1 Chest X-ray films. (**a**) (On day 85) bilateral lung fields showed a ground-glass appearance. (**b**) (On day 133) bilateral lung fields showed marked radiopacity and air bronchograms.

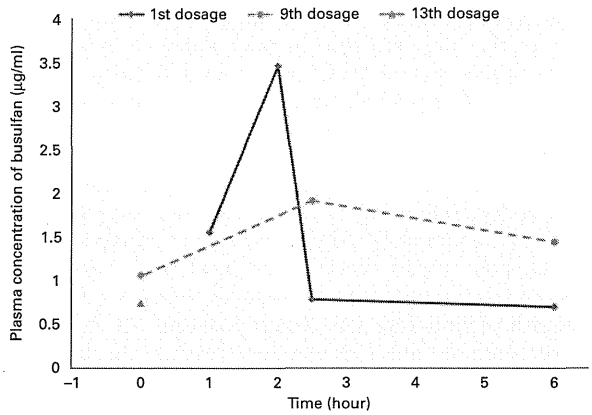

Figure 2 Plasma concentration of busulfan. Peak level of plasma concentration of busulfan is very high (1st dose). Busulfan clearance is poor (before 13th dose).

Johnson *et al.*[7] reported that the GSTA1*B variant had a 2.6-fold higher busulfan AUC level than other variants after intravenous busulfan exposure in the pediatric population. Our patient's genotype of the promoter region of GST A1 by DNA sequencing was GSTA1*A diplotype (-567T, -69C, -52G) which is thought to be more active than the GSTA1 *A/*B. Nevertheless, busulfan AUC levels were significantly elevated. This may indicate that polymorphisms other than GSTA1 polymorphisms may affect busulfan metabolism.

Intravenous busulfan should have a much more predictable pharmacokinetic profile than oral busulfan. Treatment with a fixed dose of 0.80 mg/kg intravenous busulfan achieved the target AUC level (900–1500 μM × min) in 80% of adult patients.[8] The remaining 20% were very close to achieving the target level.[8] A recent European study showed that 91% of children achieved target AUC levels by weight-based dosing.[9] They concluded that this weight-based dosing in children is sufficient without therapeutic drug monitoring and dose adjustment. Our patient received intravenous busulfan according to his body weight, as in the European study. However, his busulfan AUC levels were higher than has been reported elsewhere in the literature.[9,10] To avoid unexpectedly high busulfan AUC levels, therapeutic drug monitoring and dose adjustment should be recommended for all patients who are treated with high-dose busulfan. When therapeutic drug monitoring is not applicable, test dosing of intravenous busulfan before high-dose therapy would be preferable.

Conflict of interest

The authors declare no conflict of interest.

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References

1 Myers JL, El-Zammar O. Pathology of drug-induced lung disease. In: Katzenstein AL (ed). *Katzenstein and Askin's Surgical Pathology of Non-Neoplastic Lung Disease*, 4th edn. Elsevier Publishing Ltd: Philadelphia, PA, 2006, pp 85–89.

2 Kalaycio M, Pohlman B, Kuczkowski E, Rybicki L, Andresen S, Sobecks R *et al.* High-dose busulfan and the risk of pulmonary mortality after autologous stem cell transplant. *Clin Transplant* 2006; **20**: 783–787.
3 Henner WD, Furlong EA, Flaherty MD, Shea TC, Peters WP *et al.* Measurement of busulfan in plasma by high-performance liquid chromatography. *J Chromatogr* 1987; **416**: 426–432.
4 Li CK, Yuen PM, Wong R, Pang CP, Lai WK, Chik KW *et al.* Busulphan level and early mortality in thalassaemia patients after BMT. *Bone Marrow Transplant* 1999; **23**: 307–310.
5 Dix SP, Wingard JR, Mullins RE, Jerkunica I, Davidson TG, Gilmore CE *et al.* Association of busulfan area under the curve with veno-occlusive disease following BMT. *Bone Marrow Transplant* 1996; **17**: 225–230.
6 Czerwinksi M, Gibbs JP, Slattery JT. Busulfan conjugation by glutathione S-transferases alpha, mu and pi. *Drug Metab Dispos* 1996; **24**: 1015–1019.
7 Johnson L, Orchard PJ, Baker KS, Brundage R, Cao Q, Wang X *et al.* Glutathione S-transferase A1 genetic variants reduce busulfan clearance in children undergoing hematopoietic cell transplantation. *J Clin Pharmacol* 2008; **48**: 1052–1062.
8 Nguyen L, Leger F, Lennon S, Puozzo C. Intravenous busulfan in adults prior to haematopoietic stem cell transplantation: a population pharmacokinetic study. *Cancer Chemother Pharmacol* 2006; **57**: 191–198.
9 Vassal G, Michel G, Esperou H, Gentet JC, Valteau-Couanet D, Doz F *et al.* Prospective validation of a novel IV busulfan fixed dosing for paediatric patients to improve therapeutic AUC targeting without drug monitoring. *Cancer Chemother Pharmacol* 2008; **61**: 113–123.
10 Stein J, Davidovitz M, Yaniv I, Ben-Ari J, Gamzu Z, Hoffer E *et al.* Accidental busulfan overdose: enhanced drug clearance with hemodialysis in a child with Wiskott-Aldrich syndrome. *Bone Marrow Transplant* 2001; **27**: 551–553.

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ORIGINAL ARTICLE

Importance of *c-kit* mutation detection method sensitivity in prognostic analyses of t(8;21)(q22;q22) acute myeloid leukemia

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Recently, *c-kit* mutations have been reported as a novel adverse prognostic factor of acute myeloid leukemia with t(8;21)(q22;q22) translocation (t(8;21) AML). However, much remains unclear about its clinical significance. In this study, we developed a highly sensitive mutation detection method known as mutation-biased PCR (MB-PCR) and investigated the relationship between *c-kit* mutations and prognosis. When *c-kit* mutations were analyzed for 26 cases of t(8;21) AML using the direct sequence (DS) and MB-PCR, the latter had a much higher detection rate of *c-kit* mutations at initial presentation (DS 5/26(19.2%) vs MB-PCR 12/26(46.2%)). Interestingly for the three cases, in which *c-kit* mutations were observed only at relapse with the DS, *c-kit* mutations were detected at initial presentation using the MB-PCR. This result suggests that a minor leukemia clone with *c-kit* mutations have resistance to treatment and are involved in relapse. In univariate analyses, the presence of a *c-kit* mutation using DS was not an adverse prognostic factor ($P=0.355$), but was a factor when using MB-PCR ($P=0.014$). The presence of *c-kit* mutations with MB-PCR was also an independent adverse prognostic factor by multivariate analyses ($P=0.006$). We conclude that sensitivity of *c-kit* mutation detection method is important to predict prognosis for t(8;21) AML.

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Introduction

Acute myeloid leukemia (AML) has the highest incidence among adult leukemia and includes a variety of subtypes with varying pathology, responsiveness to treatment and prognoses.[1,2] AML with the t(8;21)(q22;q22) translocation (t(8;21) AML), constitutes about 10% of all AMLs and has been considered an AML group with favorable prognosis based on its positive responsiveness to chemotherapy and high rate of complete remission.[3–5] Many studies have reported that when high-dose Ara-C is used as post-remission therapy for t(8;21) AML, prognosis is good with overall disease-free survival rates of 50–70%.[6–11]

However, although t(8;21) AML has good prognosis overall, about 40% of cases relapse, of which half become treatment resistant. In fact, reports suggest that the prognosis of t(8;21) AML following first relapse is just as poor as other relapsed AMLs even when stem cell transplantation (SCT) is performed.[12–15] Stratifying prognoses for t(8;21) AML become critical when determining, for example, SCT indications during the initial remission period. The white blood cell (WBC) count at initial examination,[12] WBC index,[16,17] additional chromosomal aberrations such as del(9)[18] and loss of sex chromosomes and CD56 positivity[19] have been reported as adverse prognostic factors of t(8;21) AML. In recent years, *c-kit* mutations have also been proposed as a novel marker.[20,21]

The *c-kit* gene is located on chromosome 4q11–12 and encodes a 145 kD type III receptor tyrosine kinase. It has five extracellular immunoglobulin-like domains, a juxtamembrane domain and an intracellular kinase domain. *c-kit* mutations have been found in gastrointestinal stromal tumors (70% or more), mastocytosis (90% or more) and germ cell tumors (about 10%).[22,23] In addition, they have been reported in ~12–25% of cases of core-binding factor (CBF)–AMLs such as inv(16)(p13q22) and t(8;21) AML.[24] Normal *c-kit* sends proliferative cues into cells following stimulation by its ligand, stem cell factor and has an important role in early hematopoiesis.[25] On the other hand, mutant *c-kit* send proliferative signals into cells in a ligand-independent manner, and are considered class I aberrations, which have a role in leukemic cell proliferation.[26] In recent years, it has been reported that CBF–AML cases with *c-kit* mutations show a high rate of relapse and poor prognosis.[20,21,27–29] Reflecting these reports, the National Comprehensive Cancer Network's (NCCN) 2009 guidelines considered t(8;21) AML with *c-kit* mutations to be an intermediate prognosis group. However, at present, much remains unclear about the significance of *c-kit* mutations as a prognostic factor for CBF–AML. For example, in inv(16)(p13q22) AML, prognosis of patients with or without mutation in *c-kit* exon 8 is not significantly different.[21] Recently it is also reported that prognosis of pediatric CBF–AML patients with or without *c-kit* mutations is not significantly different.[30]

The sensitivity of the detection method may be one reason why *c-kit* mutations remain an unclear prognostic factor for t(8;21) AML. A large majority of t(8;21) AML cases is classified as M2 by the French–American–British (FAB) Classification. Depending on the case, the proportion of leukemic cells may be less than 50%. In addition, nearly all *c-kit* mutations are heterozygous. It is possible that leukemic cells with *c-kit* mutations are quantitatively small populations at initial presentation and hence, not detectable with the direct sequence (DS) method. If the minor leukemic cells with *c-kit* mutations at initial presentation become treatment resistant and are involved in relapse, then a highly sensitive method to detect *c-kit* mutations is necessary in order to clarify the significance of *c-kit* mutations as a prognostic factor. Therefore, we used a highly sensitive mutation detection method known as mutation-biased

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PCR (MB-PCR) to detect *c-kit* mutations and investigated their significance as a prognostic factor for t(8;21) AML.

Patients and methods

Patient samples

We retrospectively analyzed 26 cases of t(8;21) AML treated at the Nippon Medical School and its affiliated facilities between January 1991 and January 2009. A search for *c-kit* mutations and *Fms-like tyrosine kinase 3 internal tandem duplications* (*Flt3 ITD*) was carried out on 26 initial diagnosis and 15 relapse cases where bone marrow or peripheral blood samples, in which 30% or more of the blast cells were available for use. Informed consents were obtained from the patients for genetic testing. This protocol was approved by our institutional review board.

Mutation analysis for c-kit and Flt3 ITD

Mononuclear cells from bone marrow or peripheral blood were isolated by density gradient centrifugation using lymphocyte separation medium (Organon, Durham, NC, USA). Genomic DNA of mononuclear cells was extracted with the QIAamp DNA Mini Kit (Qiagen, Hilden, Germany). PCR amplification of *c-kit* and *Flt3 ITD* was performed as previously described.[31,32] Mutational analysis of the extracellular domain (exons 8 and 9), transmembrane domain (exon 10), juxtamembrane domain (exon 11) and the second intracellular kinase (TK) 2 domain (exons 17 and 18) of the *c-kit* gene was carried out with PCR followed by direct sequencing. Specific sequences of primers used for PCR and sequencing are available on request. To validate sequencing results, PCR products were inserted into the pCR2.1-TOPO vector using a TOPO TA cloning kit (Invitrogen, Carlsbad, CA, USA). Recombinant plasmids isolated from 8 to 12 white colonies were sequenced.

MB-PCR for c-kit mutations using QProbe

Quenching probe (QProbe) is a fluorescent probe in that a fluorescent substance is bound to cytosine on the terminal portion of the probe, which becomes quenched on hybridization with a complementary strand. With increasing temperature, the duplex unravels at a temperature (Tm) related to the strength of the bond between the QProbe and the complementary chain, at which point the fluorescence intensity recovers. Tm analysis is a technique to determine the degree of complementation between the QProbe and target nucleic acid by measuring the change in fluorescence intensity with increasing temperature.[33,34] Here, we detected *c-kit* mutations using the QProbe method and a prototype of i-densy, a fully automated single nucleotide polymorphism genotyping systems.[34] Amplification was performed with MB-PCR for high-sensitivity detection of mutations using probes that perfectly matched the mutated form. In MB-PCR, the 3′ terminal region of the primer is established as the mutation site, and allele-specific primers for wild-type and mutant primers are used for amplification. By adjusting the length of the primer, we devised a means by which the mutant form could be amplified more than the wild-type form. Furthermore, given the correlation between the proportion of the mutant form and the value of the fluorescence intensity detected by QProbe, it is possible to semiquantify the amount of mutations.

For sensitivity analysis, normal cells and clinical samples with heterozygous mutations were mixed and detection sensitivity was assessed. The ratio of each mutation was controlled by adjusting the ratio of blast cells from the clinical samples. Detection sensitivity was also assessed using a mixture of DNA from a normal subject and *c-kit* mutations containing fragment generated by PCR and TA cloning from clinical samples.

Statistical analysis

Patient characteristics were compared using Chi-square, Fisher and *t* tests. In addition, we analyzed the cumulative incidence of relapse (CIR) using the Kaplan–Meier log-rank test. Prognostic factors for t(8;21) AML were analyzed with multivariate analyses using Cox proportional hazards. In order to extract independent events, those events with a *P*-value of 0.20 or less were analyzed using the backward stepwise model selection procedure. Statistical analyses were carried out using SPSS software (version 12.1.4; SPSS Inc., Chicago, IL, USA).

Results

Clinical characteristics of patients with t(8;21) AML

The clinical characteristics of patients are shown in Table 1. The median age was 50.3 years (range, 31–72 years) with 69.2% being males. Patients were followed-up for 3–112 months after initial presentation, with a median of 34.5 months. Of the 26 total t(8;21) AML cases, 15 (57.7%) relapsed.

Behenoyl cytosine arabinoside (BHAC)-DM-based, DNR/Ara-C and IDA/Ara-C regimens were used as induction therapies for 16, 7 and 3 cases, respectively. BHAC-DM-based regimen was a response-oriented individualized chemotherapy regimen using BHAC, dounorbicin (DNR) and 6-mercaptppurine (6-MP).[35–38] All patients obtained complete remission by induction therapies. For post-remission consolidation, BHAC-based and HDAC-based regimens were administered in 14 and 12 cases, respectively. As t(8;21) AML was considered to have favorable prognosis, SCT was not given in any of the cases during the first remission period.

Evaluation of post-remission course revealed a range of 1–64 months until first relapse (median 16 months) and CIR of 45.2% at 1 year and 58.6% at 6 years. We further analyzed the impact of post-remission therapy, WBC counts at initial examination, WBC index and CD56 expression on relapse. However, post-remission therapies, initial WBC counts, WBC index and CD56 expression were not significantly associated with relapse, and therefore, remain of unclear prognostic significance. Specifically, BHAC-based regimen had CIR of 31.9% at 1 year and 48.9% at 6 years, whereas HDAC-based regimen resulted in CIR of 58.3% at 1 year and 66.7% at 6 years ($P=0.384$). When initial WBC counts were equal to or greater than 20 000/μl, CIR was 60.0% at 1 year and 80.0% at 6 years. When initial WBC counts were less than 20 000/μl, CIR was 36.2% at 1 year and 48.4% at 6 years ($P=0.4343$). WBC index were equal to or greater than 20, CIR was 43.2% at 1 year and 54.1% at 6 years. When WBC index were less than 20, CIR was 33.3% at 1 year and 66.7% at 6 years ($P=0.7569$). CD56 expression less than 10% was associated with CIR of 25.0% at 1 year and 52.4% at 6 years, whereas CD56 expression greater or equal to 10% resulted in CIR of 57.7% at 1 years and 66.2% at 6 years ($P=0.083$).

Comparison of chromosome analysis at initial presentation and at relapse

There were 14/26 cases (53.8%), in which a chromosomal aberration other than t(8;21)(q22;q22) was observed at initial presentation (Table 2). Among these, the most frequent was the

Table 1 Clinical and molecular characteristics of t(8;21)AML patients

Characteristics	*Total*	*MB-PCR method*		
	t(8;21)AML n = 26	*Wild type n = 14*	*c-kit mutation(+) n = 12* (*D816V: 4, N822K: 8*)	P
Male/female	18/8	10/4	8/4	0.673
Age (years)				
Median (range)	50.3 (31–72)	50.2 (31–72)	50.4 (33–67)	1.000
<60	22	12	10	
≧60	4	2	2	
PS ≧2	1	1	0	
WBC (/μl)				
Median (range)	12858 (1400–42800)	12962 (1400–42800)	12745 (3900–29900)	0.928
<20 000	21	11	10	
≧20 000	5	3	2	
Hemoglobin (g/dl)				
Median (range)	7.48 (3.2–12.5)	6.73 (3.2–10.1)	8.36 (4.2–12.5)	0.148
≧10	21	13	8	
<10	5	1	4	
Platelet ($\times 10^4/\mu l$)				
Median (range)	2.26 (0.7–8.5)	2.43 (0.7–8.5)	2.08 (0.4–5.8)	0.654
% PB blast	49.8 (10.0–93.5)	58.7 (15.5–92.0)	48.2 (10.0–93.5)	0.770
Median (range)				
% BM blast				
Median (range)	64.5 (30.4–95.0)	65.3 (37.6–95.0)	63.5 (30.4–90.0)	0.832
WBC index				
Median (range)	70.6 (9.8–296.2)	67.4 (9.8–296.2)	74.8 (19.1–163.8)	0.792
<20	3	2	1	
≧20	23	12	11	
Extramedullary involvement	—	—	—	
CD56+				
<10%	13	9	4	0.238
≧10%	13	5	8	
Flt3 ITD				
Negative	24	13	11	
Positive	2	1	1	
Relapse rate (%)	57.7%	35.7%	83.3%	0.021
CIR				
Median	18 months	Not reached	11 months	0.014
%CIR at 1 year	45.2%	22.1%	72.5%	
%CIR at 6 years	58.6%	34.4%	81.7%	

Abbreviations: AML, acute myeloid leukemia; CIR, cumulative incidence of relapse; WBC, white blood cell.

loss of a sex chromosome, which was observed in 12 cases (46.2%) (−Y: 11 cases, −X: 1 case) (Table 2).

Of 15 relapsed cases, three were unacceptable for chromosomal analyses. Additional chromosomal aberrations were observed at relapse in 10/12 cases (83.3%), which was higher relative to the rate at initial presentation. New chromosomal aberrations were observed in 9/12 cases (75%). Furthermore, most of these cases were observed with two or more chromosomal aberrations in addition to t(8;21)(q22;q22). This suggests greater chromosomal instability at relapse compared with initial presentation. However, prognostic analyses did not show a significantly higher relapse rate (RR) for cases with additional chromosomal aberrations at initial examination ($P = 0.400$). Specifically, cases with additional chromosomal aberrations at initial examination had CIR of 30.4% at 1 year and 53.6% at 6 years, whereas those without additional chromosomal aberrations at initial examination had CIR of 63.3% at 1 year and 6 years.

c-kit mutations detected by direct sequence analysis

We screened for *c-kit* mutations using DS on 26 initial presentation cases and 13 relapsed cases with t(8;21) AML, other two relapsed cases were not available for analysis. In initial presentation subject samples, mutations were observed in 5/26 cases (19.2%; three cases of D816V and two cases of

Table 2 Chromosomal aberrations, direct sequence and MB-PCR analysis of *c-kit* gene at initial presentation and relapse

	%Blast	Chromosome analysis	Direct sequence	MB-PCR
At initial presentation				
1	67.4%	46,XY,t(8;21)(q22;q22)	D816V	D816V
2	57.8%	45,X-Y,t(8;21)(q22;q22)	D816V	D816V
3	66.8%	46,XX,t(8;21)(q22;q22)	D816V	D816V
4	46.5%	46,XY,t(8;21)(q22;q22)	Wild	D816V
5	55.0%	46,XY,t(8;21)(q22;q22)	Wild	N822K
6	67.6%	45,X-Y,t(8;21)(q22;q22)	N822K	N822K
7	48.8%	45,X-Y,t(8;21)(q22;q22)	N822K	N822K
8	48.4%	46,XX,t(8;21)(q22;q22)	Wild	N822K
9	30.4%	46,XY,t(8;21)(q22;q22)	M541 L	N822K
10	80.0%	46,XX,t(1;13;21;8)(q21;q14;q22;q22)	Wild	N822K
11	90.0%	46,XX,t(8;21)(q22;q22)	M541 L, K546 K, L862 L	N822K
12	32.4%	45,X-Y,t(8;21)(q22;q22)	Wild	No signal
13	69.2%	45,X-Y,t(8;21)(q22;q22)	Wild	No signal
14	49.6%	45,X-X,t(8;21)(q22;q22)	Wild	No signal
15	37.6%	45,X-Y,t(8;21)(q22;q22)	Wild	No signal
16	63.2%	46,XY,t(8;21)(q22;q22)	M541 L	No signal
17	90.0%	46,XY,t(8;21)(q22;q22)	Wild	N822K
18	62.8%	45,X-Y,t(8;21)(q22;q22)	Wild	No signal
19	54.8%	45,X-Y,t(8;21)(q22;q22)	Wild	No signal
20	95.0%	45,X-Y,t(8;21)(q22;q22)	Wild	No signal
21	44.2%	45,X-Y,t(8;21)(q22;q22)	Wild	No signal
22	70.0%	46,XX,t(8;21)(q22;q22)	Wild	No signal
23	95.0%	46,XX,t(8;21)(q22;q22)	Wild	No signal
24	79.8%	46,XY,t(8;21)(q22;q22)	Wild	No signal
25	62.6%	46,XX,t(8;13;21)(q22;q14;q22)	Wild	No signal
26	55.1%	45,X-Y,t(8;21)(q22;q22)	Wild	No signal
At relapse				
1	75.4%	46,XY,t(8;21)(q22;q22),t(1;2)(q42;q21) 46,XY,t(8;21;22)(q22;q22:q11)	D816V	D816V
2	82.4%	45,X-Y,t(8;21)(q22;q22),t(1;13)(p36;q14),del(11)(q23) 45,X-Y,t(8;21)(q22;q22),add(7)(q22)	D816V	D816V
3	42.0%	not acceptable	D816V	D816V
4	91.5%	45,X-Y,t(8;21)(q22;q22),t(1;22)(q21;q;q13) 45,X-Y,t(8;21)(q22;q22),add(2)(q33)	D816V	D816V
5	91.8%	45,X-Y,der(2)del(2)(p21)del(2)(q33),der(8)t(8;21)(q22;q22)t(8;14)(q22;q13),add(9)(p22),add(10)(q22),add(11)(q23),der(14)t(8;21)t(8;14),add(15)(q11),add(20)(q13),der(21)t(8;21)	D816V	D816V
6		Continuous first remission		
7	55.6%	45,X-Y,t(8;21)(q22;q22),add(2)(q21)	N822K	N822K
8	87.5%	46,XX,t(8;21)(q22;q22)	N822K	N822K
9	74.7%	46,XY,6q-,7q+,-11,t(8;21)(q22;q22),+mar	M541 L	No signal
10	42.2%	46,XX,t(1;13;21;8)(q21;q14;q22;q22)	wild	No signal
11	55.0%	46,XX,t(8;21)(q22;q22)	M541 L, K546 K, L862 L	No signal
12	30.2%	Not acceptable	Wild	No signal
13	70.2%	45,X-Y,t(8;21)(q22;q22),12q-	Not acceptable	Not acceptable
14	30.0%	45,X-X,t(6;10)(q15;p13),t(8;21)(q22;q22) 45,X-X,t(8;21)(q22;q22),add(16)(q24)	Wild	No signal
15	8.0%	Not acceptable	Not acceptable	Not acceptable
16	35.2%	45,X-Y,t(8;21)(q22;q22),t(11;12)(p13;q24.3) 45,X-Y,t(8;21)(q22;q22),t(9;12)(q22;q13),t(11;12)(p13;q24.3)	M541 L	No signal
17		Drop out		
18		Continuous first remission		
19		Continuous first remission		
20		Continuous first remission		
21		Continuous first remission		
22		Continuous first remission		
23		Continuous first remission		
24		Continuous first remission		
25		Continuous first remission		
26		Continuous first remission		

N822K). In relapsed subject samples, mutations were observed in 7/15 cases (46.7%; five cases of D816V and two cases of N822K, two cases were not acceptable). All *c-kit* mutations localized to the A-loop of exon 17. Intriguingly, of the relapsed cases with *c-kit* mutations, three cases (two cases of D816V and one case of N822K) were not detected *c-kit* mutation at initial presentation. Sequences of the detected c-kit mutations were D816V (GAT→GTC) and N822K (AAT→AAG). In addition to the above-mentioned mutations, we observed three cases of M541L (ATG→CTG; 11.5%), one case of K546K (AAA→AAG; 3.8%), and one case of L862L (CTG→CTC; 3.8%) as genetic polymorphisms (Table 2).

Establishment of a highly sensitive c-kit mutation detection method with MB-PCR

There were three cases, in which *c-kit* mutations were observed only at relapse using DS. For this reason, we surmised that minor clone of leukemic cells with *c-kit* mutations at initial presentation had resistance to treatment and became involved in relapse. To prove this hypothesis, we established a highly sensitive method for detecting these *c-kit* mutations called MB-PCR. The

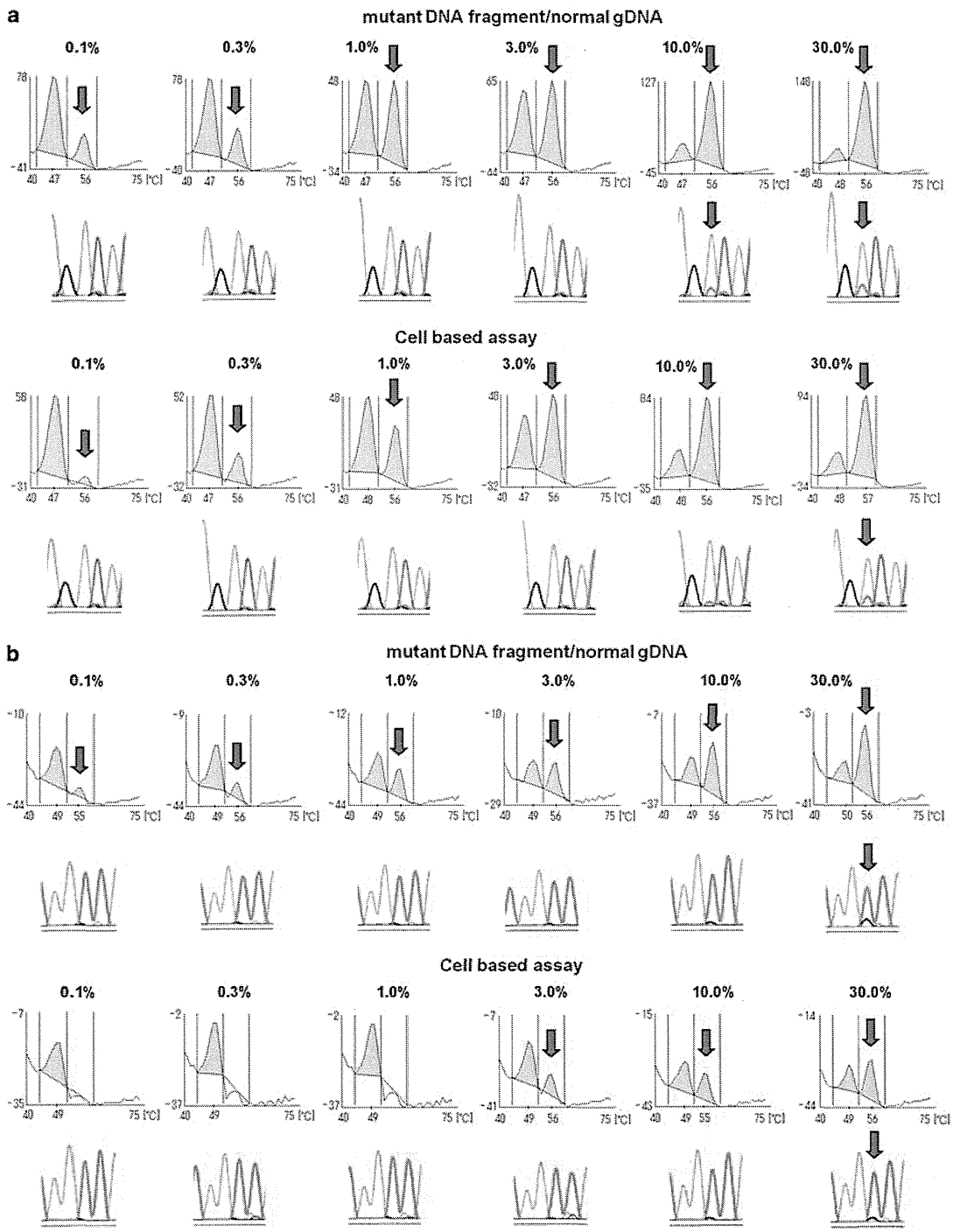

Figure 1 Comparison of detection sensitivities for c-kit exon 17 mutations. (**a**) Detection sensitivity for the D816V mutation. (**b**) Detection sensitivity for the N822K mutation. In (**a**) and (**b**), the upper panel shows the sensitivity analysis using a mixture of a mutated fragment and DNA from a normal subject. The lower panel shows sensitivity analysis using a mixed sample containing cells harboring mutations and normal cells. Sensitivity analysis was conducted using samples corresponding to 0.1, 0.3, 1, 3, 10 and 30% dilutions. The upper row in each panel shows results from MB-PCR. In contrast to wild-type *c-kit*, for which the complementary strand and QProbe separate at Tm values of 42–52 °C (D816V region) and 44–52 °C (N822K region), separation is observed at Tm values of 52–60 °C and 52–61 °C, respectively, for D816V and N822K mutations. The lower row of each panel shows results from the DS method. Arrows indicate waves of mutations.

sensitivity of detecting mutations using this method was tested by mixing cells containing D816V or N822K mutations with normal cells, which showed detection sensitivities of around 0.1% for D816V and 3.0% for N822K. Sensitivity analysis with a D816V- and N882K-containing fragment mixed with DNA from normal subjects produced a detection sensitivity of around 0.1% for both D816V and N822K. In contrast, the same sample subjected to the DS method resulted in a detection sensitivity of 10–30% for D816V and 30% for N822K. These results suggest that compared with the DS method, MB-PCR can detect mutations with much higher sensitivity (Figure 1).

Screening for c-kit mutations using highly sensitive mutation detection methods

When *c-kit* mutations of D816V and N822K were screened using MB-PCR, 12/26 of initial presentation samples were positive (46.2%; 4 cases of D816V and 8 cases of N822K). In 15 of relapse samples, two of them were not available for analyses, and seven of them were detected *c-kit* mutations (46.7%; five cases of D816V and two cases of N822K). In other words, MB-PCR offered a higher detection rate for *c-kit* mutations at initial presentation samples compared with DS.

MB-PCR was able to detect *c-kit* mutations at initial presentation or early stage for the three cases, in which *c-kit* mutations were observed only at relapse using DS. MB-PCR could detect *c-kit* mutations at initial presentation of case number 4 and 8, as well as at the first relapse of case number 5, for which *c-kit* mutations could not detected using DS (Figure 2).

Although less prevalent than the D816V and N822K mutations, there has been reports of small deletion-type mutation in the T417–D419 region of exon 8 in *c-kit* (i.e., del419, del418–419 and Thr417Ile/del418–419). In order to analyze this exon 8 mutation, we generated a QProbe (QProbe $^{T417-D419}$) directed at the wild-type sequence. However, the T417–D419 deletion could not be detected in first presentation and relapsed t(8;21) AML samples.

Clinical significance of c-kit D816V and N822K mutations

The clinical progression of the 11 cases with detected *c-kit* mutations are shown in Figure 3 (one case was not included due to a short observation period). Five cases with the D816V observed at initial presentation using the MB-PCR method all eventually relapsed, and at relapse D816V remained positive in all cases (Figure 3). For cases number 4 and 5 in particular, a low number of leukemic cells had D816V at initial presentation, which apparently became treatment resistant and amplified in subsequent relapse. Of the seven cases with N822K at initial presentation, six cases (86%) relapsed. Among these, however, N822K was observed in only two cases (33.3%) at relapse (Figure 3).

Significance of c-kit mutation as a prognostic factor in t(8;21) AML

Using DS and MB-PCR, we examined whether initial possession of a *c-kit* mutation has prognostic significance given the above MB-PCR results. No significant differences were found in clinical characteristics of case samples analyzed by both methods between those with or without *c-kit* mutations (Table 1 and Supplementary Table 1). By DS, there was a tendency for high RRs in the *c-kit* mutation (+) group, but this was not statistically significant (Figure 4a). Specifically, the *c-kit* mutation (+) group had a RR of 80.0% and CIR of 60.0% at 1 year and 80.0% at 6 years, while the *c-kit* mutation (−) group had a RR of 52.4% and CIR of 35.7% at 1 year and 52.8% at 6 years ($P=0.355$). However, with MB-PCR, the *c-kit* mutation (+) group had a statistically significant higher RR (Figure 4b). Specifically, the *c-kit* mutation (+) group had a RR of 83.3% and CIR of 72.5% at 1 year and 81.7% at 6 years, whereas the *c-kit* mutation (−) group had a RR of 35.7% and CIR of 22.1% at 1 year and 34.4% at 6 years ($P=0.014$).

Significance of c-kit mutation and Flt3ITD as a prognostic factor in t(8;21) AML

Flt3 ITD is observed in about 25% of all AMLs and is one of the gene mutations considered to be an adverse prognostic factor. However, among the 26 cases of t(8;21) AML examined, *Flt3 ITD* was observed in only 2/26 (7.7%) cases at initial presentation, which is a lower incidence relative to *c-kit* mutations. In one case, *Flt3 ITD* and *c-kit* mutations were observed simultaneously. All patients with *Flt3 ITD* demonstrated relapse after short remission period. Considering the *Flt3 ITD* (+) cases and/or MB-PCR *c-kit* mutation (+) cases together as the mutation (+) group, its relapse rate was shown to be significantly higher compared with those lacking both mutations (mutation (−) group: RR 30.8%, 1 year CIR 15.4%, 6 year CIR 34.2% versus mutation (+) group: RR 84.6%, 1 year CIR 74.8%, 6 year CIR 83.2%, $P=0.005$) (Figure 4c).

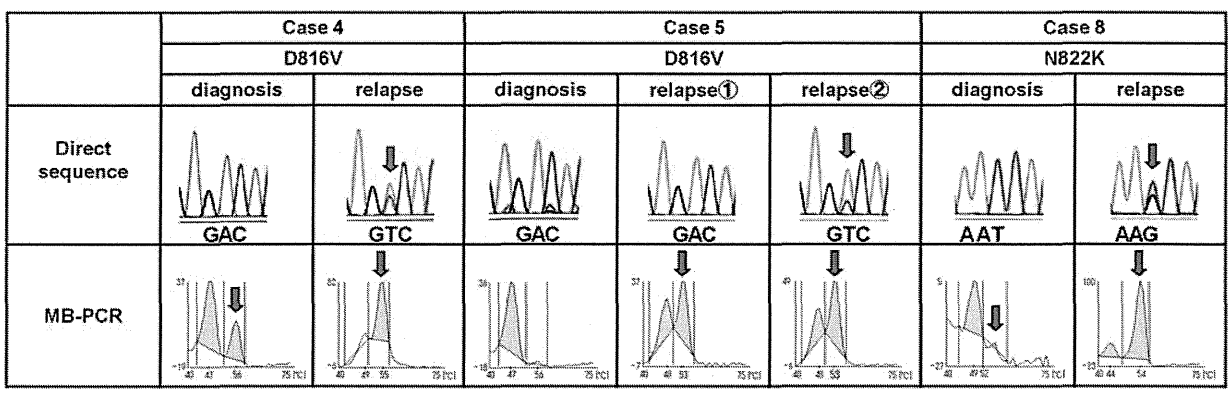

Figure 2 Detection of *c-kit* mutations with MB-PCR but not DS. The top and bottom panels, respectively, show results of the DS method and MB-PCR. For cases 4 and 8 at diagnosis and case 5 at first relapse, *c-kit* mutations were detected with MB-PCR but not with DS. Arrows indicate waves of mutations.