the following criteria: recipient gender, age at the time of transplant (0–4 yrs, 5–9 yrs, and ≥10 yrs), cytogenetics/gene mutation risk, disease status, and graft type [18].

Statistical Analysis

Pair-wise comparisons of patient characteristics (covariates) were performed using the Fisher's exact test for categorical variables. Survival was estimated using Kaplan–Meier and log-rank tests, while the cumulative incidence between groups was analyzed using Gray's test. Risk factors associated with relapse, NRM, LFS, or OS and the conditioning groups were assessed using a multivariate Cox proportional-hazard model or a competing-risks regression model. NRM was the competing event for relapse, and relapse was the competing event for NRM. STATA 12 (Stata Corp, Texas, USA) and EZR data analyses programs were used for statistical analysis. Statistical significance was set at a P value of <0.05. The present study was equipped with 73% power to detect a 20% difference in the 3-year survival rate between the RTC and MAC groups with an α error (two-sided) of 0.05. The study was approved by the institutional review boards of Matsushita Memorial Hospital and by the JSHCT committee.

RESULTS

Patient Characteristics

Thirty-four RTC transplant recipients were matched to 68 TBI-MAC and 34 Bu-MAC transplant recipients as described above. A relatively high dose of Mel was used for about half of the RTC recipients ($\geq$140 mg/m^2 for 31/34 recipients and >150 mg/m^2 for 16/34 recipients). In the Flu/Mel-RTC group, 11 patients (32%) received cytarabine or etoposide in addition to Mel, and nine patients (26%) received low-dose TBI ($\leq$4 Gy; Table I). For certain patients, the RTC regimen was primarily chosen by physicians to avoid risk of late complications (n = 10) or NRM after standard MAC (comorbidity) (n = 7). The reasons why RTC was chosen for the remaining 17 patients were not described. Among the 34 patients in the RTC group, there were two (6%) with high-risk cytogenetics/gene mutation (the RTC group *vs.* The MAC group, $P = 0.41$), although there were 53/448 patients (12%) in the MAC cohort before performing the pair-matching. Table II summarizes the patient characteristics, comorbidity, and transplant procedures in each conditioning group. Patient demographics and cytogenetic/gene mutation risk in the Flu/Mel-RTC group were comparable with those in the MAC group. The median age at transplantation was 8 years in each group. However, MAC recipients were more likely to have received HCT in the earlier half of the study period, to have received cyclosporine-based GVHD prophylaxis, and to have had a low hematopoietic cell transplantation comorbidity index score [19]. For recipients in CR2, the median interval from diagnosis to transplant was 27 and 17 months in the Flu/Mel-RTC and MAC groups, respectively, and the median follow-up time was 35 months in each group.

The actuarial incidence of engraftment, bacterial infections in the acute period, lung complications (acute respiratory distress syndrome, interstitial pneumonia, bronchiolitis obliterans organizing pneumonia, and bronchiolitis obliterans), sinusoidal obstruction syndrome and/or thrombotic microangiopathy, and grades II–IV acute and chronic GVHD did not differ in the two groups and were comparable to those of previous reports [20,21]. Cumulative 100 day incidence of NRM (day + 100 NRM: 5% in the combined group) was similar for each group (Table III). Leukemic relapse was the major cause of death (58%). The overall incidence of death from infectious complications was 2%.

Relapse and NRM after Conditioning Regimens

In the combined group, the 3-year cumulative incidence of relapse (CIR) and NRM were 25% (95% confidence interval [CI], 17–32%) and 12% (95% CI, 7–18%), respectively. In the Flu/Mel-RTC group, the 3-year CIR was 24% (95% CI, 10–41%), and in the MAC group, it was 25% (95% CI, 17–34%), with a cumulative incidence of NRM of 13% (95% CI, 4–29%) and 11% (95% CI, 6–19%) ($P = 0.73$ and $P = 0.82$) in the Flu/Mel-RTC and the MAC

TABLE III. Transplantation Outcome After RTC or MAC

	RTC		MAC		P-value	Total
	34	(%)	102	(%)		136
Engraftment					0.25	
No	1	(3)	0			1
Yes	33	(97)	102	(100)		135
Lung complication[a]					0.73	
No	31	(91)	86	(84)		117
Yes	2	(6)	9	(9)		11
UE/missing data	1	(3)	7	(7)		8
SOS/TMA					0.28	
No	29	(85)	89	(87)		118
Yes	4	(12)	6	(6)		10
UE/missing data	1	(3)	7	(7)		8
Acute GVHD						
Grade 0	12	(35)	24	(24)		36
Grade I	7	(21)	34	(33)		41
Grade II	3	(9)	22	(22)		25
Grade III	7	(21)	16	(16)		23
Grade IV	3	(9)	6	(6)		9
UE/missing data	2	(6)	0	—		2
Acute GVHD					0.84	
Grade 0–I	19	(56)	58	(57)		77
Grade II–IV	13	(38)	44	(43)		57
UE/missing data	2	(6)	0	—		2
Chronic GVHD					0.80	
No	18	(53)	60	(59)		78
Limited	4	(12)	11	(11)		15
Extensive	5	(15)	22	(22)		27
UE/missing data	7	(21)	9	(9)		16
Day+100 NRM					0.68	
No	33	(97)	96	(94)		129
Yes	1	(3)	6	(6)		7

RTC, reduced-toxicity conditioning; MAC, myeloablative conditioning; UE, unevaluable; SOS, sinusoidal obstruction syndrome; TMA, thrombotic microangiopathy; GVHD, graft-versus-host disease; NRM, non-relapse mortality. [a]including acute respiratory distress syndrome, interstitial pneumonia, bronchiolitis obliterans organizing pneumonia and bronchiolitis obliterans.

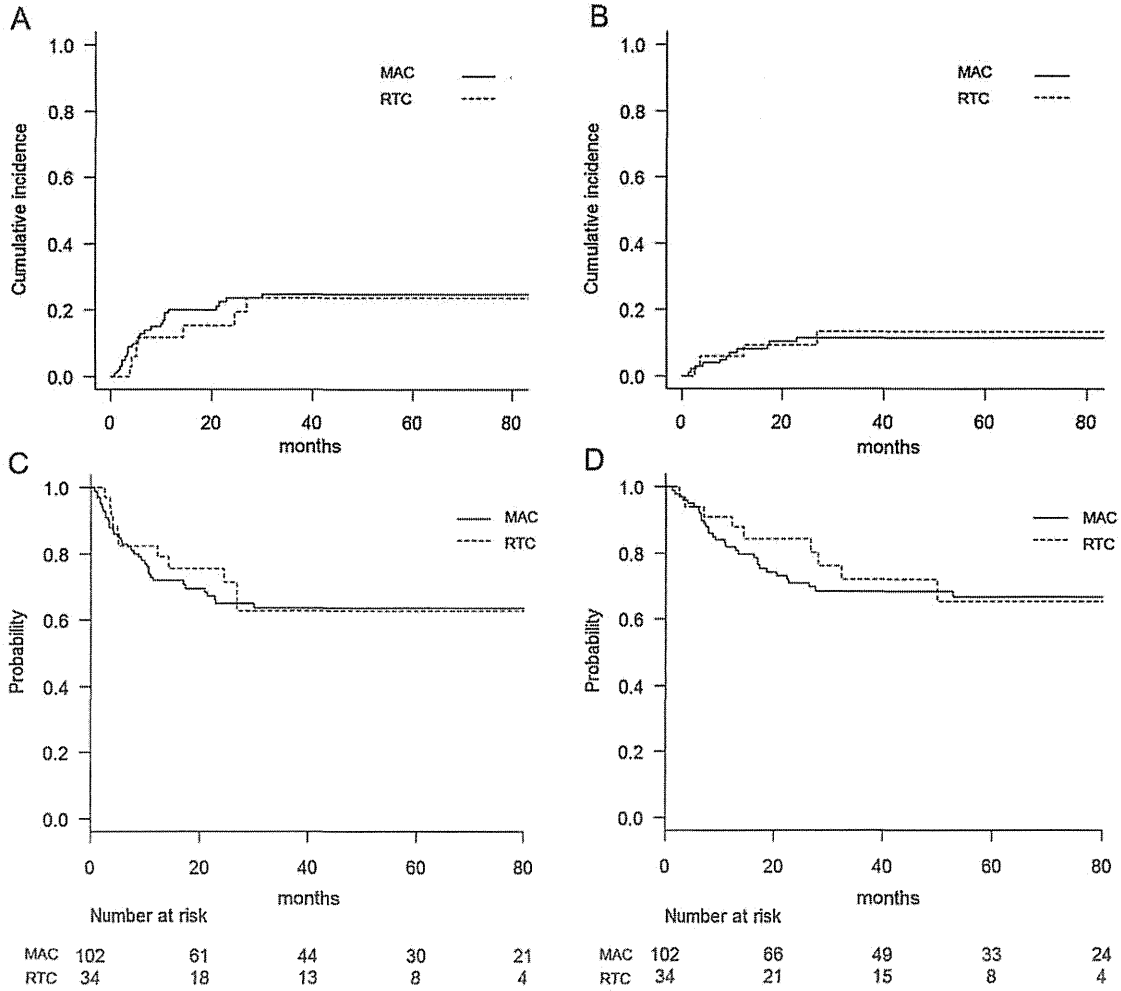

Fig. 1. (A) Cumulative incidence of relapse (CIR). The 3-year CIR in the group (n = 34) that received fludarabine (Flu)/melphalan (Mel)-based reduced toxicity conditioning (Flu/Mel-RTC: dotted line), and in the group (n = 102) that received myeloablative conditioning (MAC: solid line), were 24% (95% CI, 10–41%) and 25% (95% CI, 17–34%), respectively ($P = 0.73$). (B) Cumulative incidence of non-relapse mortality (NRM). The 3-year cumulative incidence of NRM in the RTC and MAC groups were 13% (95% CI, 4–29%) and 11% (95% CI, 6–19%), respectively ($P = 0.82$). (C) Probability of leukemia-free survival (LFS). The 3-year LFS rate in the RTC and MAC groups were 63% (95% CI, 42–78%) and 64% (95% CI, 53–72%), respectively ($P = 0.83$). (D) Probability of overall survival (OS). The 3-year OS rate in the RTC and MAC groups were 72% (95% CI, 51–85%) and 68% (95% CI, 58–77%), respectively ($P = 0.82$).

groups, respectively (Fig. 1A and B). Relapse differed significantly according to age at transplant ($P = 0.04$), despite the risk of NRM being similar for all variables (Table IV). There was no significant association between the incidence of relapse and age at transplant ($P = 0.16$) or poor performance status ([PS] ≥ 1) ($P = 0.06$) by multivariate analysis. There was also no significant association between the incidence of NRM and all variables by multiple analysis (Table IV).

LFS and OS After Conditioning Regimens

In the combined group, the estimated 3-year LFS and OS rates were 64% (95% CI, 54–71%) and 69% (95% CI, 60–77%), respectively. The 3-year LFS rate after Flu/Mel-RTC and MAC was 63% (95% CI, 42–78%) and 64% (95% CI, 53–72%), respectively ($P = 0.83$), and OS was 72% (95% CI, 51–85%) and 68% (95% CI, 58–77%), respectively ($P = 0.82$) (Fig. 1C and D). LFS and OS differed significantly according to age at transplant ($P = 0.03$ for both) (Table IV).

Multivariate analysis resulted in no significant differences in the LFS and OS rates in the Flu/Mel-RTC and MAC groups (Table IV).

Gonadal Function, Fertility in Female Recipients, and Secondary Malignancies

For technical reasons, we did not have access to adequate long-term follow-up data related to late complications. Of the 30 female recipients who survived at least 1 year after HCT and lived beyond 12 years of age (mean menarche age for Japanese females), spontaneous menstruation developed in two of seven recipients of Flu/Mel-RTC and three of 23 recipients of MAC ($P = 0.57$). None of the nine female recipients who survived beyond 20 years of age

TABLE IV. Univariate and Multivariate Analysis

	Relapse[a]		NRM[a]		LFS		OS	
Variables	% (95% CI) at 3 y	*P* value	% (95% CI) at 3 y	*P* value	% (95% CI) at 3 y	*P* value	% (95% CI) at 3 y	*P* value
Univariate[b]								
Conditioning		0.73		0.82		0.83		0.82
RTC	24 (10–41)		13 (4–29)		63 (42–78)		72 (51–85)	
MAC	25 (17–34)		11 (6–19)		64 (53–72)		68 (58–77)	
	Relapse[a]		NRM[a]		LFS		OS	
Variables	HR (95% CI)	*P* value	HR (95% CI)	*P* value	HR (95% CI)	*P* value	HR (95% CI)	*P* value
Multivariate[c]								
MAC	1		1		1		1	
RTC	0.89 (0.36–2.21)	0.80	1.00 (0.27–3.66)	1.00	0.90 (0.41–1.96)	0.80	0.74 (0.32–1.71)	0.49

y, years; NRM, non-relapse mortality; LFS, leukemia-free survival; OS, overall survival; CI, confidence interval; RTC, reduced-toxicity conditioning; MAC, myeloablative conditioning; HR, hazard ratio. [a]Cumulative incidence of relapse or NRM; [b]Univariate analysis: No significant covariates were found for NRM. A significant covariate for relapse, LFS, and OS was age at transplant ($P = 0.044$, $P = 0.025$, and $P = 0.026$, respectively); [c]Multivariate analysis: No significant covariates were found for relapse, NRM, LFS, or OS.

experienced pregnancy. No secondary malignancies developed in the RTC and matched MAC recipients.

DISCUSSION

Previous reports suggest that the relapse rate is significantly higher in adults with AML recieving a transplant in CR after RTC than after MAC [6,9]. A retrospective non-randomized study showed that, compared with MAC, RTC was associated with a lower NRM rate but a higher incidence of relapse in the recipients with adult AML and MDS, which resulted in a comparable OS rate [5,6,9]. In adult recipients with AML or MDS with non-advanced disease, intermediate intensity conditioning regimens (*e.g.*, Flu and Mel, 80–140 mg/m^2) have nearly identical short- and long-term disease progression compared with the MAC regimens [22]. As the incidence of NRM after MAC is often lower in pediatric patients than it is in adults [14,23], the effect of RTC of lowering the rate of NRM would be predicted to have less impact on the pediatric OS rates. Therefore, retaining the myeloablative intensity is more important factor than reducing the short-term comorbidity for its successful outcomes in pediatric patients who can tolerate standard MAC.

The present study compared the impact of a Flu/Mel-RTC regimen that included neither full-dose TBI nor Bu, with MAC regimens in children and adolescents with AML. The incidence of relapse after Flu/Mel-RTC was comparable to that after MAC, suggesting that the combination of Flu/Mel-RTC and allo-HCT may lead to the same anti-leukemic effect as MAC and allo-HCT. Other multicenter studies report 27–30% CIR in remission in pediatric recipients with AML who received transplantation after MAC [21,24,25]. Although the 3-year CIR of 26% found in the present study is in agreement, our finding that the rate of relapse after Flu/Mel-RTC was similar to that of MAC could be due to the fact that Mel has a potentially superior anti-AML effect compared with intravenous Bu 6.8 mg/kg (Mel 100–140 mg/m^2) [26] or Cy [27]. Also, the myeloablation caused by conditioning regimens, including Flu combined with Mel (140 mg/m^2), is not thought to be fundamentally different from that caused by MAC regimens [28], and conditioning regimens including Mel at a dose >150 mg/m^2 are thought to be myeloablative (in the RTC group, approximately half of the patients were given Mel at doses of ≥150 mg/m^2) [29]. Interestingly, it can be inferred that more than 50% of the patients in the RTC group would have been able to tolerate MAC regimens, as they had no pretransplant comorbidities to account for the choice of RTC. However, it is also reasonable to predict that patients receiving Flu/Mel-RTC had some favorable prognostic factors (*e.g.*, a low level of minimal residual disease or a longer median interval from diagnosis to transplantation in the CR2 setting in the RTC group, suggesting a longer CR1), which could have contributed to a better outcome. Also, it is possible that physicians might choose MAC regimens for patients with HiR disease in light of the low proportion of HiR patients in the RTC group. Although the proportion of those with HiR in the RTC and MAC groups, before and after performing pair-matching, did not differ significantly, the small number of patients defined as HiR in the RTC group made any evaluation difficult.

Our observations on relapse, NRM, and OS after Flu/Mel-RTC and MAC were consistent with those in childhood AML reported by Bitan et al. [14], who showed that relapse rates were not higher after RTC compared with MAC (39% *vs.* 39%), and the recipients of MAC were not at higher risk of NRM compared with recipients of RTC (16% *vs.* 16%). Accordingly, the 5-year OS rate with the RTC and MAC regimens were 45% and 48%, respectively. Similar results were achieved in Japanese patients after Flu/Mel-RTC or MAC who received the AML99 or AML-05 protocol as the initial treatment in most instances. The study reported by Bitan et al. [14], and the present study, suggested that poor PS was a predictor for relapse, although in the latter, the association did not reach statistical significance. The present study may have been underpowered to detect a significance association because the number of patients with PS >2 (n = 4) was insufficient.

The follow-up period was not sufficient to allow us to compare the incidence of late complications in the two groups. However, previous reports indicated that Flu/Mel-RTC could be associated with lower gonadal toxicity and a higher proportion of patients with conserved fertility compared with MAC [7,30]. Long-term follow-up studies of HCT following RTC for pediatric patients with leukemia would be warranted.

Taken together, the results of the present study and those of Bitan et al. suggest that in childhood and adolescent patients with AML who would be condsidered able to tolerate MAC in CR1 or CR2, a myeloablative RTC regimen is a possible alternative to a standard MAC regimen (such as a combination of TBI or Bu and another alkylator).

This registry-based study has several limitations in addition to being a retrospective analysis. First, we did not have information about gene mutations other than FLT3-ITD, and data on FLT3-ITD were missing for many cases. Second, we had no information on the minimal residual disease status, which, prior to transplantation, is a strong prognostic factor for AML in morphologic CR [31]. Third, definitive conclusions on the efficacy of each conditioning regimen could not be drawn due to the limited number of patients in the RTC group, possible bias on the choice of conditioning regimen, and differences in supportive care provided by individual physicians. Nevertheless, the present data strongly support the concept that RTC, such as a combination of Flu and Mel, deserves further evaluation in a prospective trial, as development of a conditioning regimen with effective myeloablative intensity but minimal comorbidity is being eagerly pursued for use in pediatric and adolescent patients with AML.

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Comparison of Continuous and Twice-Daily Infusions of Cyclosporine A for Graft-Versus-Host-Disease Prophylaxis in Pediatric Hematopoietic Stem Cell Transplantation

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Background. Cyclosporine A (CsA) is used widely for graft-versus-host disease (GVHD) prophylaxis in hematopoietic stem cell transplantation (HSCT); however, the optimal schedule of its administration has not been established. Although comparative studies of adult patients undergoing HSCT have demonstrated enhanced efficacy and safety of twice-daily infusion (TD) compared with continuous infusion (CIF) of CsA, to our knowledge, similar studies have not yet been performed in pediatric groups. ***Procedure.*** A self-administered questionnaire was used to retrospectively compare the clinical outcome and incidence of CsA-associated adverse events of 70 pediatric acute myelogenous leukemia patients who were receiving CsA by TD (n = 36) or CIF (n = 34) as GVHD prophylaxis for their first allogeneic HSCT. ***Results.*** The cumulative incidences of grade II–IV acute GVHD and chronic GVHD, as well as the overall survival and event-free survival rates, did not differ significantly between the TD and CIF groups; however, the incidence of severe hypertension was significantly higher in the CIF group than the TD group. ***Conclusions.*** The analysis presented here indicates that TD and CIF administration of CsA have similar prophylactic effect on pediatric GVHD and suggest that TD is associated with a lower rate of toxicity than CIF in pediatric patients undergoing HSCT. Pediatr Blood Cancer 2015;62:291–298. © 2014 Wiley Periodicals, Inc.

Key words: cyclosporine; graft-versus-host disease; hematopoietic stem cell transplantation; pediatric

INTRODUCTION

The immunosuppressive drug cyclosporine A (CsA), which is usually combined with short-term treatment with methotrexate (MTX), is used widely for the prophylaxis of graft-versus-host disease (GVHD). Traditionally, CsA is typically administrated intravenously in the early period after allogeneic hematopoietic stem cell transplantation (HSCT), after which the treatment is converted to oral administration [1].

Target CsA concentrations of 250–450 ng/ml are widely accepted for continuous infusion (CIF) of CsA [2]: however, these concentrations are not sufficient to prevent GVHD in adult patients undergoing HSCT. Although CIF of CsA at higher target concentrations (450–550 ng/ml) is more effective at preventing GVHD, these concentrations are associated with adverse effects, including hypertension and acute nephrotoxicity [3,4]. The immunosuppressive effect of CsA, which occurs via calcineurin inhibition, is concentration-dependent rather than time-dependent and its greatest pharmacodynamic effect occurs within the first 2 or 3 hr after exposure [5,6]. Hence, twice-daily infusion (TD) of CsA is used during renal, liver, and heart transplantation to reduce the occurrence of graft rejections [7].

TD administration of CsA with peak concentration monitoring has also been employed as an optimized GVHD prophylaxis regimen for adult patients undergoing HSCT [8–10]. However, the dose, target blood level, and mode of intravenous infusion vary

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among transplant institutions, and the optimal schedule of CsA administration has not yet been established. Furthermore, the comparative studies of the efficacy of various modes of CsA treatment have not yet been performed in pediatric HSCT despite of the wide use of both TD and CID modes. Therefore, the aim of this study was to evaluate the efficacy and safety of the TD and CIF modes of CsA administration for the treatment of pediatric HSCT. For this aim, we analyzed the data of pediatric patients with acute myelogenous leukemia (AML) as a single disease entity, which is one of the most popular pediatric hematological malignancies.

MATERIALS AND METHODS

Study Design and Data Collection

Using data for patients with AML provided by the Transplant Registry Unified Management Program (TRUMP) [11], which includes data from the Japan Cord Blood Bank Network (JCBBN) and the Japan Society for Hematopoietic Cell Transplantation (JSHCT), the following criteria were used to select candidates for the self-administered questionnaire: (i) children with a diagnosis of AML who were younger than 18 years old; (ii) children in which allogeneic transplantation was performed for the first time during January 2006 and December 2009; (iii) children administered CsA for GVHD prophylaxis; and (iv) children administered CsA for more than 28 days after the first transplant. The data were extracted from the database in the Japan Society for Stem Cell Transplantation Registry and 99 cases from 58 institutions were selected as candidates. The questionnaire was distributed to gather additional information about the mode of CsA administration, the daily dose; the blood concentration of CsA; and CsA-associated adverse effects, including hypertension, renal toxicity, hyperglycemia, hyperbilirubinemia, thrombotic microangiopathy (TMA), hepatic veno-occlusive disease of liver (VOD), and encephalopathy. Of the 58 transplant institutions surveyed, 44 (75.9%) responded and data for 70 patients with AML were included in the study. This study was approved by the Data Management Committee of the Nationwide Survey of the JSHCT, and the institutional ethics committees of Kyoto University Hospital and Nagoya University Hospital.

Based on the recommendation outlined in a previous report [12], myeloablative conditioning (MAC) was classified as a regimen including at least 5 Gy of total body irradiation (TBI) as a single fraction, at least 8 Gy or TBI in fractionated doses, or oral or intravenous administration of busulfan at doses greater than 8 mg/kg. All other conditioning regimens were classified as nonmyeloablative reduced intensity conditioning (RIC). For transplantation using related bone marrow (BM) or peripheral blood (PB), or unrelated cord blood (CB), HLA matching was assessed using serological data for the HLA-A, HLA-B, and HLA-DR loci. For transplantation using unrelated BM, HLA matching was assessed using allelic data for HLA-A, HLA-B, and HLA-DRB1.

Endpoints

The primary endpoint of this study was to compare the cumulative incidences of grade II-IV and grade III-IV acute GVHD, and CsA-associated adverse events between the TD and CIF groups. Other endpoints were to compare the overall survival (OS) and event-free survival (EFS) rates, and the cumulative incidences of chronic GVHD, non-relapse mortality (NRM), and relapse between the TD and CIF groups. Acute and chronic GVHD was diagnosed and graded by the attending physicians of each hospital according to the consensus criteria [13,14]. Hypertension, renal toxicity, hyperglycemia, and hyperbilirubinemia were evaluated using the National Cancer Institute's Common Terminology Criteria for Adverse Events (version 4.0), and severe adverse events were defined as grade 2 and higher. Diagnosis of VOD, TMA, and encephalopathy were made based on characteristic clinical findings, positive laboratory data, or positive radiological findings by the attending physicians at each hospital.

Statistical Analysis

The characteristics of patients in the TD and CIF groups were compared using Fisher's exact test for categorical variables and two-sample Wilcoxon's test for continuous variables. OS and EFS rates were estimated using the Kaplan–Meier method [15], and the groups were compared using the log-rank test. The cumulative incidences of grade II–IV acute GVHD, chronic GVHD, NRM, and relapse were estimated, and the groups were compared using the log-rank test. Competing events were engraftment failure, relapse, or NRM without GVHD for acute and chronic GVHD, death without relapse for relapse, and relapse for NRM. To determine prognostic factors associated with the development of grade II–IV acute GVHD and chronic GVHD, log-rank test and a Cox regression test were used. The following variables were examined in the univariate analysis: mode of CsA administration, patient age, sex match, stage of AML, HSCT type, ABO match, conditioning regimen, and CMV serostatus. Factors with $P < 0.2$ in log-rank tests were included in the Cox regression model. To determine prognostic factors associated with the development of severe hypertension, Fisher's exact test and a logistic regression test were used. The following variables were examined in the univariate analysis: mode of CsA administration, patient age, occurrence of grade 1 hypertension before HSCT, use of melphalan (Mel), use of ≥ 8 Gy of TBI, conditioning regimen, use of prednisolone or methylprednisolone for GVHD prophylaxis and/or treatment, and HSCT type. Factors with $P < 0.2$ in Fisher's exact tests were included in the logistic regression model. All statistical analyses were performed using Stata software (version 12; StataCorp, TX). The authors had full access to the data and assume responsibility for their integrity. The P values were two-sided and $P < 0.05$ was considered significant for all analyses.

RESULTS

Characteristics of the Patients

Of 70 pediatric patients with AML, 36 (51.4%) and 34 (48.6%) received TD and CIF of CsA, respectively. The characteristics of the patients and the associated clinical data are listed in Table I. Most of the patients received MAC (58 of 70 patients; 82.9%), and most underwent short-term treatment with MTX in combination with CsA (63 of 70 patients; 90.0%). Prednisolone was administrated to only two patients (2.9%). There were no significant differences between any of the baseline characteristics of the TD and CIF groups (Table I). The median time to switch to oral administration of CsA in the TD and CIF groups were 41 days (range, 20–73 days) and 36 days (range, 21–84 days), respectively. In the TD group, CsA was administrated over two (n = 15), three (n = 19), four (n = 1), or

TABLE I. Characteristics of the 70 Patients Included in the Study

Variable	TD (n = 36)	%	CIF(n = 34)	%	*P*-value
Recipient age (years), median (range)	9 (0–17)		10 (1–17)		0.120
Patient sex					
Male	20	55.6	20	58.8	0.813
Female	16	44.4	14	41.2	
Sex match					
Match	15	41.7	13	38.2	0.323
Male to female	10	27.8	5	14.7	
Female to male	7	19.4	13	38.2	
Missing	4	11.1	3	8.8	
Diagnosis					
M0	0	0	2	5.9	0.663
M1	5	13.9	7	20.6	
M2	8	22.2	8	23.5	
M3	0	0	1	2.9	
M4	5	13.9	3	8.8	
M5a	7	19.4	2	5.9	
M5b	0	0	1	2.9	
M6	1	2.8	1	2.9	
M7	7	19.4	6	17.6	
With MD	1	2.8	2	5.9	
Others	2	5.6	1	2.9	
De novo					
De novo	33	91.7	31	91.2	1.000
Secondary	3	8.3	3	8.8	
WBC at diagnosis (/μl), median (range)	19,700 (1,300–405,900)		9,750 (610–290,000)		0.428
Stage					
1CR	21	58.3	17	50.0	0.562
2CR	3	8.3	6	17.6	
NCR	12	33.3	11	32.3	
HSCT type					
MR-BM/CB	15	41.7	13	38.2	0.641
MR-PB	3	8.3	7	20.6	
MMR-BM/PB	4	11.1	4	11.8	
MU-BM	2	5.6	2	5.9	
U-CB	12	33.3	8	23.5	
ABO match					
Matched	23	63.9	18	52.9	0.811
Minor mismatched	4	11.1	6	17.6	
Major mismatched	4	11.1	4	11.8	
Major-minor mismatched	5	13.9	6	17.6	
Conditioning regimen					
MAC	31	86.1	28	82.4	0.750
RIC	5	13.9	6	17.6	
GVHD prophylaxis					
+MTX	34	94.4	29	85.3	0.153
+PSL	0	0	1	2.9	
+MTX, PSL	1	2.8	0	0	
CsA alone	1	2.8	4	11.8	
CMV serostatus					
Negative donor to negative patient	5	13.9	4	11.8	0.924
Positive donor to negative patient	2	5.6	2	5.9	
Negative donor to positive patient	6	16.7	4	11.8	
Positive donor to positive patient	12	33.3	15	44.1	
Unknown	11	30.6	9	26.5	
Follow-up (days), median (range)	700.5 (56–1,599)		567.5 (69–1,409)		0.282

MD, myelodysplasia; WBC, white blood cell; 1CR, first complete remission; 2CR, second complete remission; NCR, no complete remission; MR-BM/CB, HLA-matched related bone marrow/cord blood; MR-PB, HLA-matched related peripheral blood stem cells; MMR-BM/PB, HLA-mismatched related bone marrow/peripheral blood stem cells; MU-BM, HLA-matched unrelated bone marrow; U-CB, unrelated cord blood; MAC, myeloablative conditioning; RIC, reduced intensity conditioning; MTX, methotrexate; PSL, prednisolone.

five hours (n = 1). None of the patients underwent *in vivo* or *ex vivo* T cell depletion.

Treatment Outcome

The median follow-up duration was 590.5 days (range, 56–1599 days). The OS (Fig. 1A) and EFS (Fig. 1B) rates did not differ significantly between the TD and CIF groups. Furthermore, there were no significant differences in the cumulative incidences of grade II–IV acute GVHD (Fig. 1C) and chronic GVHD (Fig. 1D) between the TD and CIF groups. The differences in the cumulative incidences of grade II–IV acute GVHD or chronic GVHD were also not significant when the dataset was limited to patients treated with CsA and MTX (data not shown). There were no significant differences in the cumulative incidence of grade III–IV acute GVHD between the TD and CIF groups (grade III–IV acute GVHD at day 100: TD group, 0%; CIF group, 3.0 ± 3.0%; $P = 0.303$). The cumulative incidences of relapse and NRM did not differ

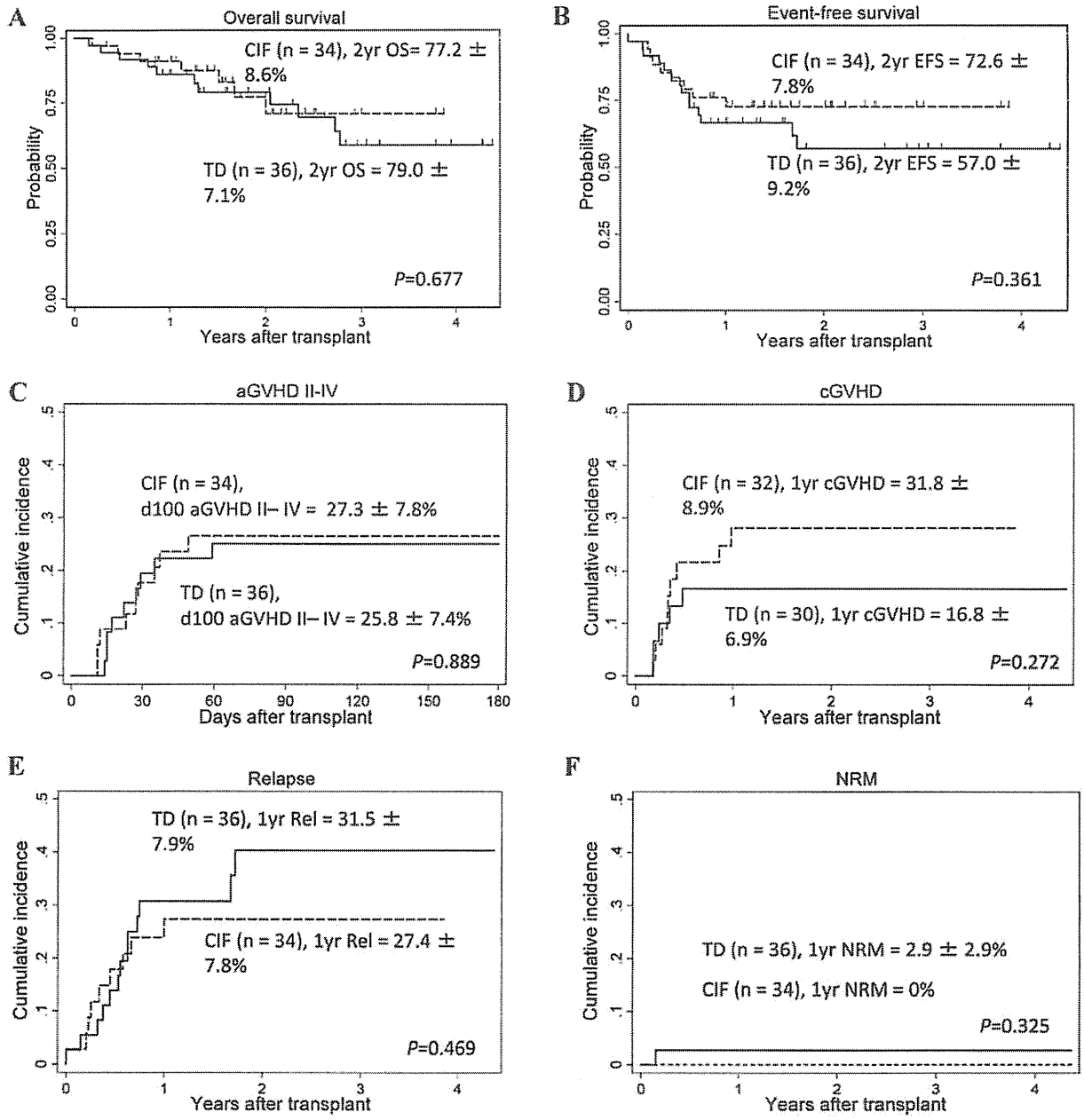

Fig. 1. The overall survival (A) and event-free survival (B) rates, and the cumulative incidences of grade II–IV acute GVHD (C) and chronic GVHD (D) among patients grouped by the mode of CsA administration. The cumulative incidences of relapse (E) and non-relapse mortality (F) among patients grouped by the mode of CsA administration. The solid and dashed lines indicate the TD and CIF groups, respectively.

significantly between the TD and CIF groups (Fig. 1E and F). Next, a univariate analysis was performed to evaluate the impact of potential confounding factors on the development of grade II–IV acute GVHD. Stage of AML, HSCT type, and CMV serostatus were identified as risk factors for grade II–IV acute GVHD; however, a multivariate analysis using a Cox regression test demonstrated that no independent risk factors were identified (Table II). For chronic GVHD, stage of AML, HSCT type, and conditioning regimen were identified as risk factors; however, a multivariate analysis demonstrated that no independent risk factors were identified.

Incidence of CsA-associated Adverse Events

The incidences of CsA-associated adverse events in the TD and CIF groups during the first 28 days after transplantation were compared. For each adverse event, patients who had grade 2 or higher toxicity before transplantation were excluded from the analysis. The incidence of severe hypertension was significantly higher in the CIF group than the TD group; however, the incidences of severe renal toxicity, hyperglycemia, and hyperbilirubinemia, TMA, VOD, and encephalopathy did not differ significantly between the two groups (Table III).

Univariate and Multivariate Analyses of Factors Related to the Development of Severe Hypertension

Univariate analysis was performed to evaluate the impact of potential confounding factors on the development of severe hypertension. As shown in Table IV, CIF administration of CsA, grade 1 hypertension before HSCT, the use of melphalan, and conditioning regimen were identified as risk factors for severe hypertension. A multivariate analysis using a logistic regression test was then performed to identify independent risk factors for the development of severe hypertension. CIF administration of CsA was identified as the sole independent significant risk factor.

Daily Doses and Trough Blood Concentration of CsA

In a previous study of adult patients receiving CsA, the incidence of grade II–IV acute GVHD was significantly higher and renal toxicity was significantly less frequent in the CIF group than the TD group [8]. In the adult study, patients in the TD group received a higher dose of CsA than those in the CIF group and the trough blood concentrations in these two groups were maintained at 150–300 ng/ml and 250–400 ng/ml, respectively [8]. To enable a direct comparison of the results, the daily doses and trough blood

TABLE II. Univariate and Multivariate Analyses of the Effects of Pre-transplantation Factors on the Incidence of Grade II-IV Acute GVHD in the 70 Patients Included in the Study

				Multivariate analysis	
Characteristics	Factors (n)	Grade II-IV acute GVHD	Univariate analysis *P*-value	Odds ratio (95% CI)	*P*-value
CsA mode	TD (36)	27.3 ± 7.8	0.889	1.02 (0.40–2.58)	0.973
	CIF (34)	25.8 ± 7.4			
Age group	0–9 (34)	21.2 ± 7.1	0.284	N.E.	N.E.
	10–17 (36)	31.4 ± 7.9			
Sex match	Match (28)	25.0 ± 8.2	0.294	N.E.	N.E.
	Male to female (15)	40.7 ± 12.9			
	Female to male (20)	27.8 ± 10.6			
	Missing (7)	0			
Stage	1CR (38)	15.8 ± 5.9	0.015	1.58 (0.95–2.63)	0.075
	2CR (9)	55.6 ± 16.6			
	NCR (23)	33.3 ± 10.3			
HSCT type	MR-BM/CB (28)	25.0 ± 8.2	0.011	1.00 (0.75–1.34)	0.987
	MR-PB (10)	0			
	MMR-BM/PB (8)	62.5 ± 17.1			
	MU-BM (4)	66.7 ± 27.2			
	U-CB (20)	21.0 ± 9.4			
ABO match	Matched (41)	20.6 ± 6.5	0.455	N.E.	N.E.
	Minor mismatched (10)	40.0 ± 15.5 %			
	Major mismatched (8)	37.5 ± 17.1			
	Major-minor mismatched (11)	27.3 ± 13.4			
Conditioning regimen	MAC (59)	28.1 ± 6.0	0.536	N.E.	N.E.
	RIC (11)	18.2 ± 11.6			
CMV serostatus	Negative donor to negative patient (9)	11.1 ± 10.5	0.159	1.19 (0.80–1.70)	0.390
	Positive donor to negative patient (4)	0			
	Negative donor to positive patient (10)	30.0 ± 14.5			
	Positive donor to positive patient (27)	42.3 ± 9.7			
	Unknown (20)	15.8 ± 8.4			

N.E., not evaluated; 1CR, first complete remission; 2CR, second complete remission; NCR, no complete remission; MR-BM/CB, HLA-matched related bone marrow/cord blood; MR-PB, HLA-matched related peripheral blood stem cells; MMR-BM/PB, HLA-mismatched related bone marrow/peripheral blood stem cells; MU-BM, HLA-matched unrelated bone marrow; U-CB, unrelated cord blood; MAC, myeloablative conditioning; RIC, reduced intensity conditioning.

TABLE III. The Incidences of Complications (≥grade 2 and ≥grade 3) in Patients Grouped by the Mode of CsA Administration

Complication	CsA mode	Cases	≥grade2	*P*-value	≥grade3	*P*-value
Hypertension	TD	36	2 (5.5%)	0.021	0 (0%)	0.010
	CIF	34	9 (26.5%)		6 (17.6%)	
Hyperglycemia	TD	36	3 (8.3%)	0.466	0 (0%)	0.225
	CIF	33	5 (15.2%)		2 (6.1%)	
Renal toxicity	TD	36	6 (16.7%)	0.261	1 (2.8%)	1
	CIF	34	2 (5.9%)		1 (2.9%)	
Hyperbilirubinemia	TD	36	1 (2.8%)	0.608	0 (0%)	0.478
	CIF	33	2 (6.1%)		1 (3.0%)	

concentrations of CsA were evaluated in the 70 patients included in this study during the first 28 days after transplantation.

No significant differences in the daily doses of CsA were observed between the TD and CIF groups at days 7, 14, 21, and 28 (Fig. 2A). The trough blood concentrations of CsA in the TD group at days 7, 14, 21, and 28 were 122.9 ± 68.1 ng/ml, 158.7 ± 71.5 ng/ml, 187.2 ± 102.5 ng/ml, and 190.6 ± 93.0 ng/ml, respectively. The corresponding concentrations in the CIF group were 294.8 ± 83.3 ng/ml, 350.9 ± 138.3 ng/ml, 335.8 ± 132.3 ng/ml, and 310.5 ± 119.0 ng/ml, respectively. At days 7, 14, 21, and 28, trough concentrations of CsA below 150 ng/ml occurred in 58.3%, 52.9%, 38.2% and 31.0% of patients in the TD group, respectively. Trough concentrations below 250 ng/ml occurred in 17.6%, 14.7%, 23.5% and 30.0% of patients in the CIF group at days 7, 14, 21, and 28, respectively. These data indicate that, compared with the CIF group, a significantly higher percentage of patients in the TD group were treated with a lower dose of CsA during the first two weeks after transplantation than that reported in a previous study of CsA administration to adults undergoing HSCT[8] ($P = 0.009$ and $P = 0.002$ at days 7 and 14, respectively) (Fig. 2B).

DISCUSSION

Because uncontrolled variables, such as patient age and underlying disease, may influence the incidence or severity of acute GVHD, it is necessary to evaluate the efficacy and safety of different types of GVHD prophylaxis within homogenous groups of patients. To achieve this aim, a nationwide survey was performed to select pediatric AML cases who had recently received their first allogeneic transplantation and had been treated with CsA for GVHD prophylaxis. Historically, CsA was administered to most pediatric patients via CIF; however, the mode of CsA administration in Japan has gradually shifted to TD over the last few years. Consequently, the 70 patients selected for inclusion in this study were divided approximately equally between the TD and CIF groups, which enabled a reliable comparison of the effect of CIF and TD

TABLE IV. Univariate and Multivariate Analyses of the Effects of Pre-transplantation Factors on the Incidence of Severe Hypertension (HT) in the 70 Patients Included in the Study

				Multivariate analysis	
Characteristics	Factors (n)	≥grade2	Univariate analysis *P*-value	Odds ratio (95% CI)	*P*-value
CsA mode	TD (36)	2 (5.6%)	0.022	7.99 (1.37–46.4)	0.021
	CIF (34)	9 (26.5%)			
Age group	0–9 (34)	6 (17.6%)	0.750	N.E.	N.E.
	10–17 (36)	5 (13.9%)			
Grade 1 hypertension before HSCT	Yes (65)	9 (13.8%)	0.173	6.26 (0.69–57.2)	0.173
	No (5)	2 (40.0%)			
Mel	Yes (31)	7 (22.6%)	0.196	0.35 (0.06–1.86)	0.217
	No (39)	4 (10.3%)			
TBI ≥8 Gy	Yes (43)	6 (14.0%)	0.739	N.E.	N.E.
	No (27)	5 (18.5%)			
PSL/mPSL for GVHD prophylaxis and/or treatment	Yes (14)	4 (28.6%)	0.212	N.E.	N.E.
	No (56)	7 (12.5%)			
Conditioning regimen	MAC (59)	7 (11.9%)	0.063	3.45 (0.56–21.4)	0.182
	RIC (11)	4 (36.4%)			
SCT type	MR-BM/CB (28)	2 (7.1%)	0.325	N.E.	N.E.
	MR-PB (10)	2 (20.0%)			
	MMR-BM/PB (8)	2 (25.0%)			
	MU-BM (4)	0 (0%)			
	U-CB (20)	5 (25.0%)			

N.E., not evaluated; Mel, melphalan; TBI, total body irradiation; PSL, prednisolone; mPSL, methylprednislone; MAC, myeloablative conditioning; RIC, reduced intensity conditioning; MR-BM/CB, HLA-matched related bone marrow/cord blood; MR-PB, HLA-matched related peripheral blood stem cells; MMR-BM/PB, HLA-mismatched related bone marrow/peripheral blood stem cells; MU-BM, HLA-matched unrelated bone marrow; U-CB, unrelated cord blood.

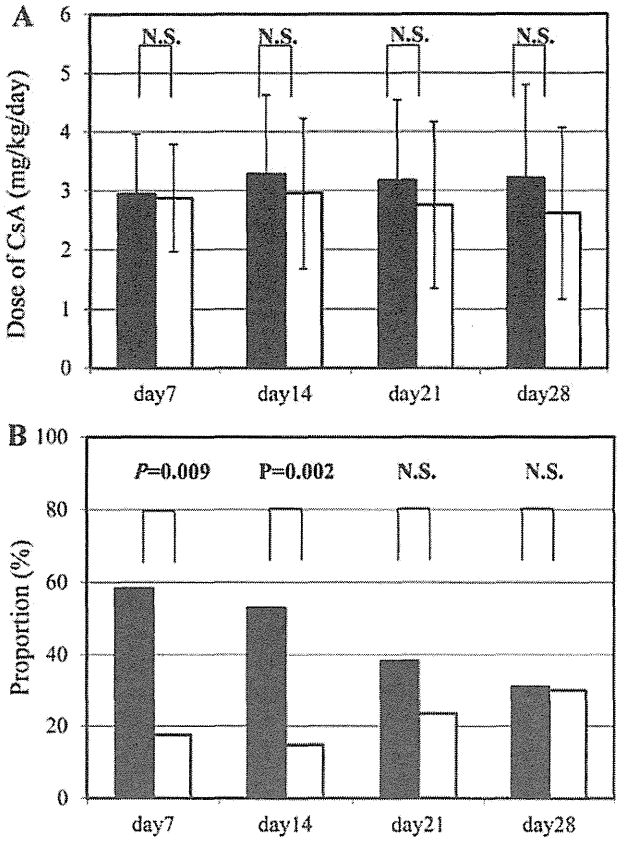

Fig. 2. (A) The daily doses of CsA administrated to patients in the TD (solid bars) and CIF (open bars) during the first 28 days after transplantation. (B) The percentages of patients in the TD (solid bars) and CIF (open bars) groups with trough concentration of CsA below 150 ng/ml (TD group) or 250 ng/ml (CIF group) during the first 28 days after transplantation. N.S., not significant. The data are presented as the mean ± SD.

administration of CsA among relatively homogenous pediatric populations.

In a previous study of adults undergoing HSCT, renal dysfunction was significantly less frequent in the CsA CIF group than the TD groups [8]. By contrast, in the current study, the incidences of CsA-associated adverse events, including renal dysfunction, were comparable in the TD and CIF groups. A possible explanation for the lack of increased renal dysfunction in the TD group observed here is that a large proportion of the pediatric TD patients (>50% in the first 14 days after transplantation) had trough concentration of CsA less than those reported in the adult study. By contrast, a significantly smaller proportion of pediatric patients in the CIF group had trough concentrations lower than those reported in the adult study. Alternatively, the pharmacokinetics and adverse effects of CsA may differ between pediatric and adult patients. Notably, CIF of CsA was identified as the sole independent risk factor for the development of severe hypertension, although TMA and encephalopathy, both of which are closely related to CsA-associated hypertension, occurred rarely in both the TD and CIF groups. Clinicopathological findings, as well as animal model studies, have indicated that CsA-induced acute reversible nephrotoxicity, caused by vasoconstriction of the afferent arterioles, might trigger the development of chronic irreversible damage to renal vessels, interstitial tubules, and glomeruli [16]. Furthermore, hypertension can persist long-term in some HSCT survivors [17], and the presence of multiple cardiovascular risk factors, including hypertension, is associated with an increased risk of late cerebrovascular disease and coronary artery disease after HSCT [18]. TD administration of CsA to pediatric patients undergoing HSCT may reduce the risk of late-occurring sequelae in long-term survivors.

Unlike a comparative previous study in adults [8], the analysis presented here fails to demonstrate the superiority of TD over CIF of CsA for the prevention of acute GVHD in pediatric patients undergoing HSCT. The lower incidence of acute GVHD in pediatric patients undergoing HSCT than adult patients undergoing HSCT, reported previously [19], may be related to similar efficiencies of different types of GVHD prophylaxis in children. Alternatively, it is possible that the peak concentrations of CsA did not reach levels sufficient to induce beneficial effects in a considerable proportion of the pediatric patients in the TD group. The limitations of this study include a retrospective analysis of small numbers of patients within the groups. Therefore, prospective randomized controlled studies are required to evaluate the efficiency and safety of TD administration alongside measurements of the peak concentration of CsA in pediatric patients undergoing HSCT.

In summary, this study demonstrates that TD is a potentially promising mode of CsA administration to pediatric HSCT patients, since the incidence of severe hypertension was lower in the TD group than the CIF group. Additional prospective studies of larger pediatric populations, including long-term follow-ups, are required to validate the efficacy and safety of TD administration of CsA.

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ORIGINAL ARTICLE

High expression of *EVI1* and *MEL1* is a compelling poor prognostic marker of pediatric AML

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EVI1 and *MEL1* are homolog genes whose transcriptional activations by chromosomal translocations are known in small subsets of leukemia. From gene expression profiling data of 130 Japanese pediatric acute myeloid leukemia (AML) patients, we found that *EVI1* and *MEL1* were overexpressed in ~30% of patients without obvious translocations of these gene loci, and that their high expression was significantly associated with inferior survival. High *EVI1* expression was detected mainly in myelomonocytic-lineage (designated as e-M4/M5 subtype) leukemia with *MLL* rearrangements and in megakaryocytic-lineage (designated as e-M7 subtype) leukemia, and its prognostic association was observed in the e-M4/M5 subtype but not in the e-M7 subtype. On the other hand, high *MEL1* expression was detected in myelocytic-lineage (designated as e-M0/M1/M2 subtype) and e-M4/M5 subtype leukemia without *MLL* rearrangements, and its prognostic association was independent from the subtypes. Because of their subtype-dependent and mutually exclusive expression, a combined evaluation of their high expression enabled a clear distinction of patients with inferior survival ($P < 0.00001$ in event-free survival (EFS) and overall survival (OS)). This association was confirmed by quantitative reverse transcription PCR analysis of an independent cohort of 81 patients ($P = 0.00017$ in EFS, $P = 0.00028$ in OS). We propose that the combined estimation of *EVI1* and *MEL1* expression will be an effective method to predict the prognosis of pediatric AML.

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INTRODUCTION

Acute myeloid leukemia (AML) is a heterogeneous disease with a variety of genetic alterations and a distinct prognosis. It has become clear that specific chromosomal abnormalities and gene mutations are its most important prognostic markers and therefore useful for stratification of patients to risk-adapted therapeutic strategies.[1,2] In pediatric AML, almost the same chromosomal abnormalities and gene mutations are observed and associated with survival as those in adult AML, while their frequencies often differ between adult and pediatric patients.[3] In a Japanese clinical study of risk-adapted therapy of pediatric AML (AML-05 study), t(8;21), inv(16)/t(16;16), monosomy 7, 5q−, t(16;21)(p11;q22), Ph1 and *FLT3*-ITD were used for patient stratification.[4] However, by such genetic alterations alone, an accurate evaluation of risk for treatment failure or relapse is still difficult.

To overcome this limitation, gene expression is a promising candidate for biomarkers to evaluate such risk. Microarray technology can quantify expression levels of tens of thousands of genes at once, and has provided many improvements in understanding the biology and subtypes of AML.[5–9] Thus, this technology was previously expected to become an effective diagnostic tool, and, in fact, several prognostically useful gene expression signatures were reported.[10–12] Recently, we also found a signature related to the *NUP98-NSD1* fusion gene to be a good candidate to detect poor prognostic patients in pediatric AML.[13] However, microarray technology has not yet spread in clinical practice, mainly because of its high cost.[9,14] On the other hand, expression levels of several genes, including *BAALC*, *MN1*, *ERG* and *EVI1* (also known as *MECOM*), have also been reported to be useful as prognostic markers.[2,15–18] Such single-gene expression markers that can be evaluated by quantitative reverse transcription (RT)-PCR should become a cost-effective tool for risk-adapted therapy.

In this study, using microarray technology, we analyzed gene expression of 130 Japanese pediatric AML patients in order to search for excellent prognostic expression markers. First, we found six subtypes that reflect chromosomal abnormalities and cell lineages, which we designated as e-t(8;21), e-inv(16), e-t(15;17), e-M0/M1/M2, e-M4/M5 and e-M7, and constructed an algorithm to classify the patients into those subtypes. Based on this classification, we found that most of the reported prognostic expression markers exhibited subtype-specific expression patterns, although some, at least in our Japanese cohort, were not prognostic. Finally, we found that a reported marker, *EVI1*, and a novel marker, *MEL1* (also known as *PRDM16*), were expressed in a mutually exclusive manner and that their combination, by working complementarily to each other, would be an excellent prognostic marker.

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MATERIALS AND METHODS

Patients and samples

One hundred thirty pediatric patients (⩽15 years old), who were diagnosed as AML between 2000 and 2002 in Japan and registered in AML99 study,[19–21] were enrolled in this study (Table 1 and Supplementary Table 1). The diagnosis was based on the French-American-British (FAB) classification, and cytogenetic analysis was performed using conventional G-banding. All leukemic samples were obtained from bone marrow or peripheral blood at the time of diagnosis, and total RNA was prepared using an RNeasy Mini Kit (Qiagen, Hilden, Germany). To detect *MLL* gene rearrangements, multiplex RT-PCR analysis[22,23] with the HemaVision kit (DNA Technology, Aarhus, Denmark) was also used. Of these 130 patients, 124 had been used and reported in our previous study.[13] Also enrolled in this study as a validation cohort were 81 patients (⩽18 years old) who were diagnosed as AML between 2006 and 2007, and were registered in AML-05 study[4] (Supplementary Table 2). Informed consent was provided from the patients or the patients' parents, according to guidelines based on the tenets of the revised Helsinki protocol and the ethical guidelines for epidemiological research in Japan (http://www.niph.go.jp/wadai/ekigakurinri/guidelines.pdf). The institutional review boards of the National Cancer Center and Gunma Children's Medical Center approved this study.

Microarray analysis

For microarray gene expression analysis, the integrity of total RNA was confirmed using a 2100 Bioanalyzer and an RNA 6000 Nano LabChip Kit (Agilent Technologies, Santa Clara, CA, USA). The DNA microarray used was a Human Genome U133 Plus 2.0 array (Affymetrix, Santa Clara, CA, USA). Target cRNA was prepared from 20 ng of total RNA with a Two-cycle cDNA Synthesis Kit and 3′-Amplification Reagents for IVT Labeling (Affymetrix). Hybridization to the microarrays, washing and staining with the antibody amplification procedure and scanning were performed according to the manufacturer's instructions. Using GeneChip Operating Software version 1.4 (Affymetrix), the scanned image data were processed and the expression value (Signal) and detection call (Present, Marginal or Absent) of each probe set were calculated. The Signal values were normalized so that the mean in each experiment was set at 100 to adjust for minor differences between the experiments. The data were deposited in Gene Expression Omnibus (accession no. GSE35784).

Table 1. Pediatric AML patients analyzed in this study (AML99 study patients)

	EVI1-overexpressed (n = *21*)	*MEL1-overexpressed* (n = *20*)	*Others* (n = *89*)	*Total* (n = *130*)
FAB subtypes				
M0	1	1	3	5
M1	3	7	10	20
M2		2	39	41
M3			13	13
M4	2	5	10	17
M5	5	3	9	17
M6	1			1
M7	7	1	5	13
Unknown	2	1		3
Karyotype/gene fusion				
t(8;21)			41	41
inv(16)			8	8
t(15;17)			13	13
11q23 (*MLL* fusion)	8		5	13
NUP98-NSD1 fusion		7		7
Others/unknown	13	13	22	48
FLT3-ITD				
Positive	1	11	6	18
Negative	20	9	77	106
Unknown			6	6

Abbreviations: AML, acute myeloid leukemia; FAB, French-American-British classification.

Quantitative RT-PCR analysis

Quantitative RT-PCR analysis was carried out using the 7900HT Fast Real Time PCR System with TaqMan Gene Expression Master Mix and TaqMan Gene Expression Assay (Applied Biosystems, Foster City, CA, USA). In addition to *EVI1* and *MEL1*, *ABL1* was evaluated as a control gene.[24] TaqMan Gene Expression Assays used for *EVI1*, *MEL1* and *ABL1* were Hs00602795_m1, Hs00922674_m1 and Hs01104728_m1, respectively. cDNA was prepared from 0.8 to 1.0 μg of total RNA using Ready-To-Go RT-PCR Beads (GE Healthcare, Buckinghamshire, UK), and 1/200 of the cDNA was used as a template for each PCR. The concentration of each transcript was determined as a molar concentration using plasmid DNA that cloned cDNA of the respective gene as a standard sample. The high or low expression of *EVI1* and *MEL1* was determined based on whether the *EVI1/ABL1* and *MEL1/ABL1* ratios of each patient were higher or lower than 0.1, respectively.

Analysis of microarray data

For statistical analysis of the microarray data, the Signal values were log transformed after the addition of 10 to reduce any adverse effect caused by noises at low expression levels. Most of the statistical analyses including hierarchical clustering analysis and Student's *t*-test were performed on GeneSpring GX software version 7.31 (Silicon Genetics, Redwood City, CA, USA) after the log-transformed expression values were normalized to the median of all patients enrolled in each of the analyses. To select appropriate probe sets defining the six subtypes of pediatric AML [e-t(8;21), e-inv(16), e-t(15;17), e-M0/M1/M2, e-M4/M5 and e-M7] (Supplementary Figure 1), representative patients of each subtype were selected (Supplementary Table 1), and Welch's *t*-tests between one and the other five subtypes were performed. Probe sets with more than threefold differences between one and the other five subtypes were then selected. Finally, the top 100 probe sets with low *P*-values in each *t*-test were totaled (Supplementary Table 3). The centroid of each subtype was determined by calculating the average of the normalized expression values for the selected probe sets. The distance (D) from a centroid to a patient was defined as $D = 1 - r$, using Pearson's correlation coefficient (r, $-1 \leqslant r \leqslant 1$; Supplementary Figure 2). Each patient is classified according to the smallest distance (D_{min}). Margin (ΔD) was defined as the difference between D_{min} and the distance to the second closest subtype to the patient (Supplementary Figure 3). When all Ds were $\geqslant 0.4$ or ΔD was < 0.05, the patient was designated as unclassified. For evaluation of *BAALC*, *MN1*, *ERG*, *EVI1* and *MEL1* expression, Signal values of probe sets 218899_s_at, 205330_at, 213541_s_at, 226420_at and 232424_at were used, respectively. Their high or low expression was determined based on a higher or lower Signal value of each patient compared with the average value of the 130 AML99 study patients, respectively.

Analysis of clinical data

All analyses were carried out using the JMP program version 8.0.1 (SAS Institute, Cary, NC, USA). Survival distributions were assessed using the Kaplan–Meier method and the differences were compared using the log-rank test. Event-free survival (EFS) and overall survival (OS) were defined as the times from diagnosis to event (relapse or death from any cause) and from diagnosis to death from any cause, respectively.

RESULTS

Pediatric AML patients can be classified into six subtypes by gene expression

Gene expression of 130 Japanese pediatric AML patients (AML99 study patients, see Materials and methods section) was analyzed with an oligonucleotide microarray. To obtain an overview and to evaluate the differences in gene expression among the 130 patients, unsupervised two-dimensional hierarchical clustering analysis was performed (Supplementary Figure 1). Patients with t(8;21), inv(16) and t(15;17) formed uniform clusters, which had been repeatedly reported.[6–8] Patients without these translocations formed different clusters, and they corresponded well to the FAB subtypes, which reflect cell lineages. Patients with FAB M7 subtype (megakaryocytic lineage) gathered into a single cluster, and patients with FAB M4 and M5 subtypes (myelomonocytic lineage) combined into a prominent cluster. Most of the other

patients with FAB M0, M1 and M2 subtypes (myelocytic lineage) loosely composed a remaining cluster. Patients with 11q23 translocations (*MLL* rearrangements) did not form a specific cluster, but most of them were distributed within a cluster of patients with FAB M4 and M5. Altogether, according to their distinct gene expression profiles, pediatric AML patients were roughly separated into six subgroups according to their karyotypes and FAB subtypes/cell lineages.

These subgroups probably reflect distinct origins and leukemogenic pathways, and may be expected to provide clinical importance. Thus, we selected genes differentially expressed among each subgroup (Supplementary Table 3) and developed an algorithm to classify pediatric AML patients into the six subtypes, which we designated as e-t(8;21), e-inv(16), e-t(15;17), e-M0/M1/M2, e-M4/M5 and e-M7 (see Materials and methods section and Supplementary Figure 2). By applying this algorithm to an original set of the 130 patients, we reclassified them based on gene expression (Supplementary Figure 3). As summarized in Supplementary Table 4, all the patients with t(8;21), inv(16) and t(15;17) were correctly reclassified, but an FAB M5 patient without t(8;21) was included in the e-t(8;21) subtype. In addition, subtypes of seven patients were changed from the original FAB subtypes. Consequently, 125 patients were separated into the six new gene expression-based subtypes, and five patients remained unclassified (Supplementary Figure 1 and Supplementary Table 4).

High expression of *BAALC*, *MN1* and *ERG* was not associated with inferior survival in Japanese pediatric AML patients

The six subtypes reclassified by our algorithm exhibited different survivals (Supplementary Figure 4). However, this subtyping alone was not enough to separate poor prognostic patients among those without major translocations of t(8;21), inv(16) and t(15;17). On the other hand, it was reported that high expression of *BAALC*, *MN1* and *ERG* was associated with inferior survival of pediatric as well as adult AML patients.[2,15,18] We examined the association between expression of these genes and survival in 130 AML99

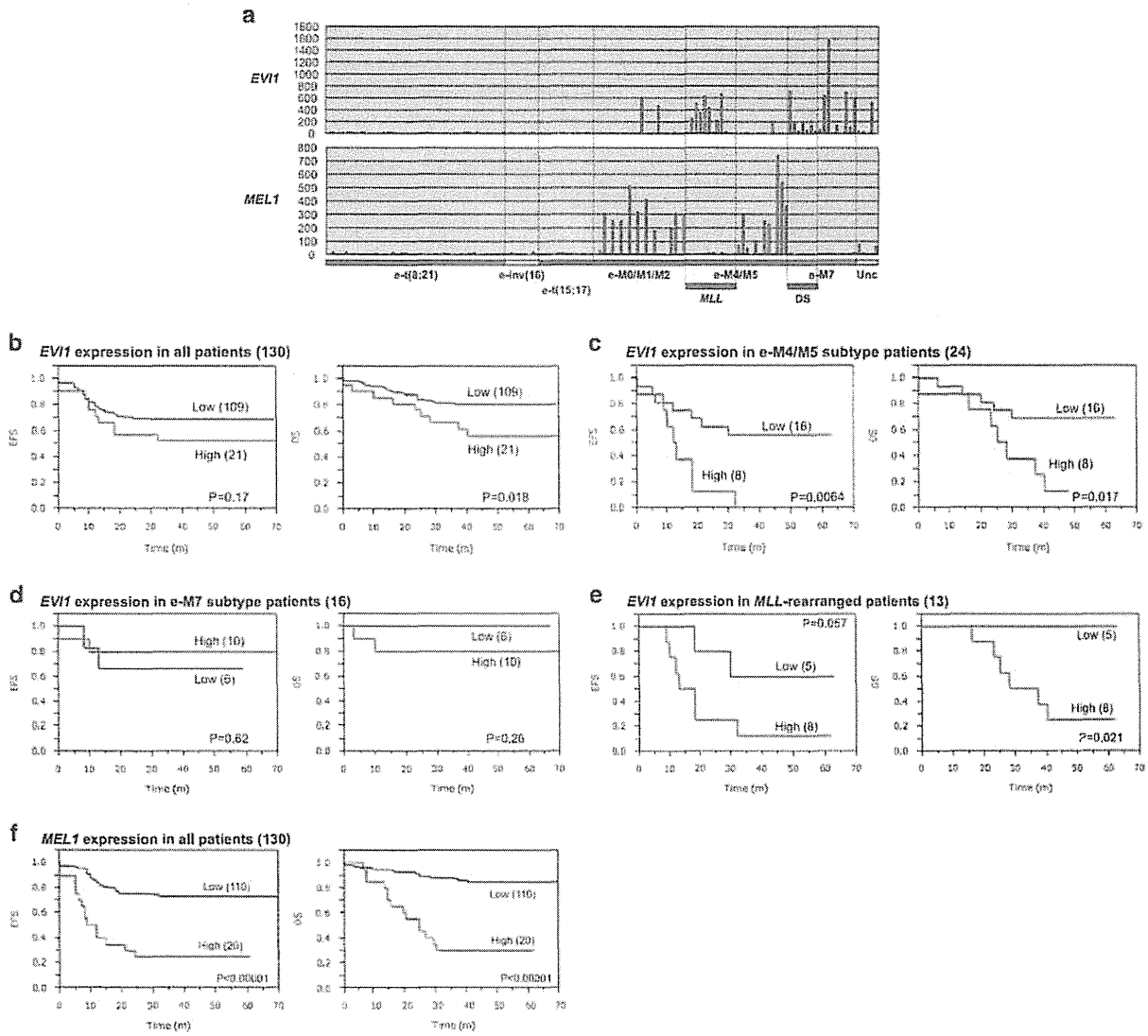

Figure 1. Mutually exclusive expression of *EVI1* and *MEL1* and their association with patient survival. (**a**) Expression levels of *EVI1* and *MEL1* (Signal values of 226420_at and 232424_at probe sets, respectively; see also Materials and methods section) in 130 AML99 study patients. Patients are arrayed according to their reclassified subtypes. (**b**–**e**) Comparison of EFS and OS between patients with high *EVI1* expression and those with low *EVI1* expression in the analyses of all patients ($n = 130$) (**b**), e-M4/M5 subtype patients ($n = 24$) (**c**), e-M7 subtype patients ($n = 16$) (**d**), and *MLL*-rearranged patients ($n = 13$) (**e**). (**f**) Comparison of EFS and OS between patients with high *MEL1* expression and those with low *MEL1* expression in the analyses of all patients ($n = 130$).

study patients. Their expression seemed to be subtype specific, but was not associated with patient survival. *BAALC* was expressed at higher levels in e-t(8;21), e-inv(16) and e-M0/M1/M2 subtypes, and the survival of patients with high expression was differently superior from the previous report[18] (Supplementary Figure 5). *MN1* was expressed highly in the e-inv(16) subtype and intermediately in the e-t(8;21) and e-M0/M1/M2 subtypes, and its expression did not affect patient survival (Supplementary Figure 6). *ERG* was almost uniformly expressed among the subtypes, but at very low levels in the e-M4/M5 subtype, and the survival of patients with high expression was relatively superior (Supplementary Figure 7). These subtype-specific expression patterns seemed to become clear when using our gene expression-based subtypes.

High expression of *EVI1* and *MEL1* was associated with inferior survival

EVI1 was another gene whose high expression was reported to be associated with inferior survival, especially in patients with *MLL* rearrangements.[16] Among the AML99 study patients, *EVI1* was overexpressed mainly in e-M4/M5 subtype patients with *MLL* rearrangements and in e-M7 subtype patients (Figure 1a). Translocations of the *EVI1* locus (3q26) were not observed in any of these patients. The survival of patients with high *EVI1* expression was slightly inferior in the analysis of total patients (Figure 1b). When the patients were divided into subtypes, inferior survival was clearly observed in the analyses of e-M4/M5 subtype patients and *MLL*-rearranged patients but not in the analysis of e-M7 patients (Figures 1c and e). It was unclear whether high *EVI1* expression was prognostic for e-M4/M5 subtype patients without *MLL* rearrangements, because such patients were very rare (Figure 1a).

MEL1 is a homolog of *EVI1*. We previously reported that the prognosis of patients with *NUP98-NSD1* fusion and its related gene expression (*NUP98-NSD1* signature) was poor by the analysis of the same cohort of patients.[13] *MEL1* was one of the representative overexpressed genes in the *NUP98-NSD1* signature. Thus, we examined *MEL1* and found that its high expression was observed in e-M0/M1/M2 and e-M4/M5 subtype patients without *MLL* rearrangements (Figure 1a), and that high *MEL1* expression was significantly associated with inferior survival in the analysis of total patients (Figure 1f). Translocations of the *MEL1* locus (1p36) were not observed in any of these patients.

Combination of high *EVI1* expression and high *MEL1* expression is a compelling poor prognostic marker

As *EVI1* and *MEL1* were expressed in a mutually exclusive manner (Figure 1a), a combination of high *EVI1* expression and high *MEL1* expression is expected to become an excellent poor prognostic marker. When 41 patients with high *EVI1* or *MEL1* expression were selected, their survival was very poor (4-year EFS: 39%, 4-year OS: 44%) and clearly different from that of patients without their high expression (hazard ratio (HR) = 3.89, $P < 0.00001$ in EFS; HR = 9.49, $P < 0.00001$ in OS; Figure 2a). In addition, when e-M7 subtype patients were excluded from the analysis, the survival of patients with their high expression was further inferior (4-year EFS: 26%, 4-year OS: 32%) and the difference became even clearer (HR = 5.63, $P < 0.00001$ in EFS; HR = 11.59, $P < 0.00001$ in OS; Figure 2b), because the survival of e-M7 subtype patients with high *EVI1* expression was not inferior (Figure 1d).

Next, we performed multivariate analyses (Table 2). High *EVI1* or *MEL1* expression, *FLT3*-ITD mutation and favorable karyotype [t(8;21), inv(16), or t(15;17)] were evaluated as independent prognostic markers with the use of a Cox regression model. Both in the analyses of all patients and in the analyses of excluding e-M7 subtype patients, high *EVI1* or *MEL1* expression was an independent poor prognostic marker for EFS and OS.

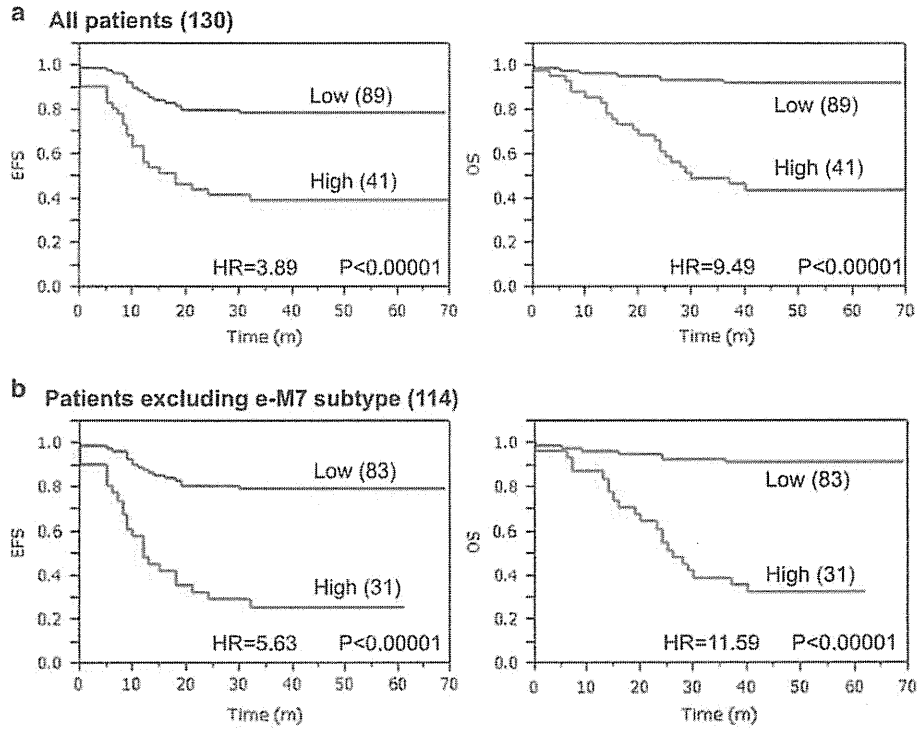

Figure 2. High expression of EVI1 or *MEL1* was associated with inferior survival. Comparison of EFS and OS between patients with high *EVI1* or *MEL1* expression and those with low *EVI1* and *MEL1* expression in the analysis of total AML99 study patients ($n = 130$) (**a**), and in the analysis excluding e-M7 subtype patients ($n = 114$) (**b**).

Table 2. Multivariate analyses of EFS and OS of AML99 study patients

	HR (95% CI)	*P-value*
EFS of all patients (n = 124)		
High *EVI1/MEL1* expression	2.55 (1.14–6.47)	0.022
FLT3-ITD mutation	2.32 (1.10–4.58)	0.029
t(8;21)/inv(16)/t(15;17)	0.64 (0.25–1.74)	0.36
OS of all patients (n = 124)		
High *EVI1/MEL1* expression	15.90 (3.27–286.32)	0.00005
FLT3-ITD mutation	2.55 (1.11–5.53)	0.028
t(8;21)/inv(16)/t(15;17)	2.12 (0.34–40.57)	0.46
EFS of patients excluding e-M7 subtype (n = 109)		
High *EVI1/MEL1* expression	4.09 (1.63–12.45)	0.0020
FLT3-ITD mutation	1.81 (0.84–3.68)	0.13
t(8;21)/inv(16)/t(15;17)	0.76 (0.27–2.40)	0.61
OS of patients excluding e-M7 subtype (n = 109)		
High *EVI1/MEL1* expression	16.82 (3.40–304.48)	0.00005
FLT3-ITD mutation	2.04 (0.87–4.58)	0.098
t(8;21)/inv(16)/t(15;17)	1.72 (0.28–33.00)	0.60

Abbreviations: CI, confidence interval; EFS, event-free survival; HR, hazard ratio; OS, overall survival. For these analyses, we used 124 and 109 patients whose *FLT3*-ITD mutation status was known.

Among the 31 overexpressed patients excluding the e-M7 subtype patients, 25 obtained complete remission (CR) of at least 6 months. Eleven patients received allogenic stem cell transplantation (allo-SCT) in first CR and the other 14 patients did not (chemotherapy only; Table 3). Eight (73%) of the 11 patients that received allo-SCT retained CR without relapse. On the other hand, only two (14%) of the 14 patients that did not receive allo-SCT retained CR. Thirteen of the 15 relapsed patients died. The relapse rates and death rates were significantly different between the presence and the absence of allo-SCT in first CR ($P = 0.0051$ and 0.049 in Fisher's exact test, respectively). Our observations suggest a possibility that SCT improves the survival of patients with high *EVI1* or *MEL1* expression.

We then examined the reproducibility of the association of high expression of *EVI1* and *MEL1* with inferior survival using an independent cohort. We used pediatric AML patients registered in AML-05 study (see Materials and methods section and Supplementary Table 2). The *EVI1* and *MEL1* expression of the AML-05 study patients was evaluated by quantitative RT-PCR and judged as overexpressed when their ratios to *ABL1* expression were >0.1 (see Materials and methods section and Figure 3a). Among the 81 AML-05 study patients that we examined, 12 overexpressed *EVI1* and 15 overexpressed *MEL1* without overlapping (Figure 3a). Two of the 12 *EVI1*-overexpressed patients were diagnosed as FAB M7 subtype. The survival of *EVI1*- or *MEL1*-overexpressed patients was significantly inferior to the others both in the analysis of all patients (HR = 3.04, $P = 0.00017$ in EFS; HR = 3.63, $P = 0.00028$ in OS; Figure 3b) and in the analysis excluding FAB M7 patients (HR = 4.32, $P < 0.00001$ in EFS; HR = 5.21, $P = 0.00001$ in OS; Figure 3c). Exclusion of FAB M7 patients seemed to improve the performance of this prognostic marker.

DISCUSSION

In this study, by analyzing gene expression profiles of 130 Japanese patients, we found that most of the pediatric AML patients were divided into six subtypes based on their gene expression, and showed that reported prognostic expression markers often exhibited subtype-specific expression patterns.

Table 3. Therapies and outcomes of *EVI1/MEL1*-overexpressed patients who obtained CR

	Total (n = 25)	*Allo-SCT (n = 11)*	*Chemotherapy (n = 14)*	*P-value*
Relapse				0.0051
CR	10 (40%)	8 (73%)	2 (14%)	
Relapsed	15 (60%)	3 (27%)	12 (86%)	
Survival				0.049
Alive	10 (40%)	7 (64%)	3 (21%)	
Dead	15 (60%)	4 (36%)	11 (79%)	

Abbreviations: allo-SCT, allogenic stem cell transplantation; CR, complete remission.

Moreover, we found that two homolog genes, *EVI1* and *MEL1*, were expressed in a subtype-dependent and mutually exclusive manner among these patients, and that their high expression was associated with inferior survival. This finding was reproduced in an independent cohort of patients. As the association with survival was extremely clear, the high expression of *EVI1* and *MEL1* is expected to be an excellent prognostic marker.

Our gene expression profiling analysis of 130 AML99 study patients revealed that pediatric AML patients were separated into six subgroups with distinct characteristics. Based on this observation, we developed an algorithm to classify patients into the six gene expression subtypes [e-t(8;21), e-inv(16), e-t(15;17), e-M0/M1/M2, e-M4/M5 and e-M7]. Although it is known that cell lineages themselves are not useful for stratification of AML patients, some prognostic markers are likely to work in a cell lineage-dependent manner. High *EVI1* expression was often observed among the e-M7 subtype patients, but was not prognostic in this subtype. In addition, some reported prognostic gene expression markers exhibited clear subtype-specific expression patterns but were not prognostic for the patients examined in this study. Thus, knowing the cell lineage of each patient correctly must still be important, which prompts us to believe that our algorithm should be useful for future researches.

The high expression of several genes such as *BAALC*, *MN1*, *ERG* and *EVI1* was reported to be associated with inferior survival of patients and prognostically useful in pediatric AML.[17,18] However, in our analysis, expression of *BAALC*, *MN1* and *ERG* was varied in subtype-specific manners, and their high expression was not associated with inferior survival. Thus, their clinical usefulness as prognostic markers may be limited, at least among Japanese patients. The survival of patients with high *EVI1* expression was inferior, but in the analysis of all patients the difference was slight (Figure 1b). A clear difference was observed only when the analysis was restricted to the patients with the e-M4/M5 subtype or to those with *MLL* rearrangements. These results suggest that for prognostic gene expression markers, considering subtypes is crucial.

Our analyses revealed that high *EVI1* expression could distinguish the poor prognostic patients among the e-M4/M5 subtype patients with *MLL* rearrangements, and that high *MEL1* expression could detect the poor prognostic patients from the e-M0/M1/M2 and e-M4/M5 subtype patients without *MLL* rearrangements. Because of their mutual exclusiveness, the combination of high *EVI1* expression and high *MEL1* expression is expected to be very effective for identification of high-risk patients. Actually, the survival of patients without their high expression was greatly superior in the analysis of the AML99 study patients (4-year EFS: 79%, 4-year OS: 92%). These findings were validated in the analysis of the AML-05 study patients, although patients without the high expression exhibited a slightly inferior

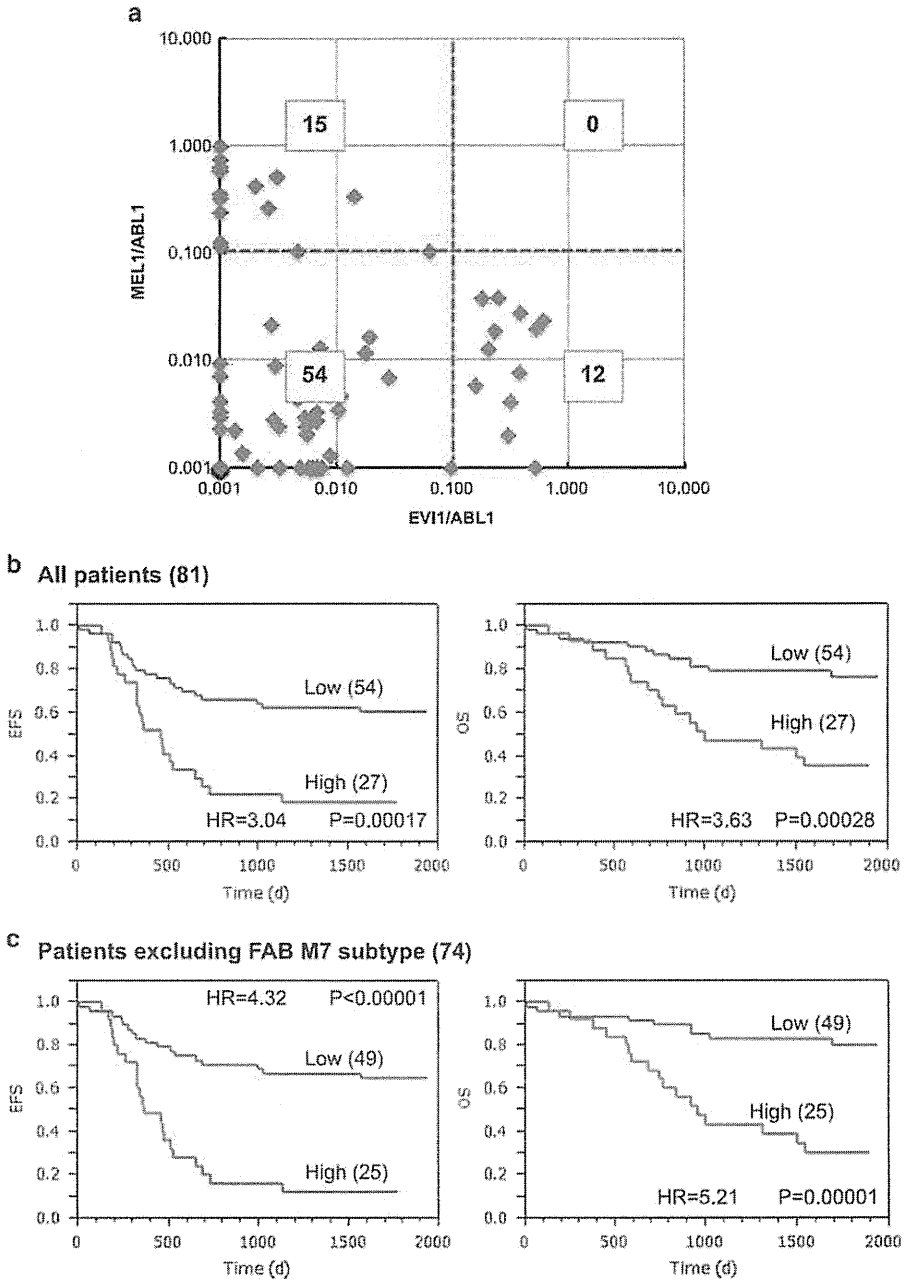

Figure 3. Validation of association between high expression of *EVI1* or *MEL1* and inferior survival. (**a**) Expression of *EVI1* and *MEL1* in 81 AML-05 study patients. Expression levels are shown as ratios of molar concentrations of mRNA determined by quantitative RT-PCR (see also Materials and methods section). (**b**, **c**) Comparison of EFS and OS between patients with high *EVI1* or *MEL1* expression and those with low *EVI1* and *MEL1* expression in the analysis of total AML-05 study patients ($n=81$) (**b**), and in the analysis excluding FAB M7 subtype patients ($n=74$) (**c**).

survival in the analysis of the AML-05 study patients (4-year EFS: 62%, 4-year OS: 79%). This is probably attributable to the lower intensity of chemotherapy to low-risk patients of the AML-05 protocol.

Among the 31 AML99 study patients with high *EVI1* or *MEL1* expression, excluding the e-M7 subtype patients, 25 obtained CR of at least 6 months but 15 relapsed and 15 died. However, their outcomes were likely affected by the therapy that they had received. The relapsed rate (27%) of the patients who had received allo-SCT was much lower than that (86%) of the patients who had not received allo-SCT (Table 3). Our observations raised the possibility that SCT improves the survival of patients with high *EVI1* or *MEL1* expression.

Both *EVI1* and *MEL1* were translocated genes in AML.[25,26] Their products have the same domain structure with high homology (63% identical in amino-acid sequences) in their entire regions.[26] It is also known that they share common/redundant biological functions. EVI1 and MEL1 are histone H3 lysine 9 monomethyltransferases that function for maintenance of heterochromatin integrity.[27] More importantly, the *Evi1* and *Mel1* genes are selectively expressed in hematopoietic stem cells in mice, and their knockouts cause defects in hematopoietic stem cells.[28–30]

Considering the lines of evidence to support causative involvement in AML and these structural and functional similarities, high expression of *EVI1* and *MEL1* is likely to activate the same oncogenic pathway and to function as a direct cause of poor prognosis. As high *EVI1* or *MEL1* expression was very tightly associated with inferior survival, this pathway should be an appropriate therapeutic target for poor prognostic patients in the future.

Our results indicate that high *EVI1* or *MEL1* expression is a compelling poor prognostic marker, and also raised the possibility that it would be useful for treatment decision. However, as this is a retrospective study, further confirmation using other patient cohorts will be necessary.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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AUTHOR CONTRIBUTIONS

Y Hayashi, TY and HI designed and organized this study; AJ, SM, NS, Y Hara and HI performed molecular analysis; Y Hayashi, IT, AT, KH, DT, TT and SA collected patient samples and clinical data; AJ, HT and HI analyzed and interpreted data; and AJ and HI wrote the manuscript.

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Supplementary Information accompanies this paper on the Leukemia website (http://www.nature.com/leu)