Table 4. Univariate and multivariate analyses of factors influencing NRM among patients transplanted in CR1, according to donor type

	Related (n = 310)					Unrelated (n = 331)				
		Univariate		Multivariate			Univariate		Multivariate	
Covariates	n	HR (95% CI)	P	HR (95% CI)	P	N	HR (95% CI)	P	HR (95% CI)	P
WBC count at diagnosis										
< 30 000/μL	224	1.00		—		230	1.00		—	
30 000/μL or more at diagnosis	86	1.21 (0.63-2.34)	.57	—		101	0.79 (0.48-1.30)	.35	—	
Lineage										
B	218	1.00		—		203	1.00		—	
T	50	1.25 (0.41-3.81)	.53	—		54	0.62 (0.29-1.38)	.17	—	
Other	42	0.87 (0.34-2.26)	.78	—		74	1.08 (0.65-1.81)	.76	—	
Karyotype										
Normal	193	1.00		—		208	1.00		—	
t(4;11) or t(1;19)	21	0.77 (0.16-3.17)	.73	—		11	1.03 (0.25-4.30)	.63	1.11 (0.27-4.64)	.57
Other (no t(9;22))	96	0.92 (0.47-1.81)	.81	—		112	1.47 (0.94-2.29)	.09	1.67 (1.06-2.64)	.03
JALSG risk stratification										
Low	39	1.00		—		45	1.00		—	
Intermediate	163	1.85 (0.86-3.97)	.12	—		192	1.01 (0.62-1.65)	.96	—	
High	108	2.82 (1.09-7.31)	.03	—		94	1.03 (0.50-2.10)	.94	—	
Age at allo-SCT										
< 45 y old	255	1.00		—		281	1.00		—	
45 y old or older at allo-SCT	55	3.90 (2.09-7.25)	< .0001	3.90 (2.09-7.25)	< .0001	50	1.26 (0.72-2.20)		.42	
HLA										
Match	285	1.00		—		192	1.00		—	
Mismatch	25	1.64 (0.64-4.18)	.30	—		139	1.69 (1.10-2.60)	.02	1.69 (1.10-2.61)	.02
Stem cell source										
Bone marrow	212	1.00		—					—	
Peripheral blood	98	1.75 (0.94-3.28)	.08	—					—	
Time from diagnosis to allo-SCT										
6 mo or longer	169	1.00		—		23	1.00		—	
< 6 mo	141	1.64 (0.87-3.11)	.13	—		308	0.31 (0.08-1.25)	.10	—	
< 10 mo	278	1.00		—		166	1.00		—	
10 mo or longer	32	1.07 (0.42-2.72)	.89	—		165	1.90 (1.21-2.99)	.01	1.98 (1.26-3.13)	.003
Preparative regimen										
Non-TBI regimens	25	1.00		—		12	1.00		—	
TBI regimens	285	0.63 (0.25-1.61)	.34	—		319	0.67 (0.25-1.85)	.44	—	
GVHD prophylaxis										
Cyclosporine A with or without other	283	1.00		—		171	1.00		—	
Tacrolimus with or without other	27	1.66 (0.65-3.80)	.29	—		160	1.33 (0.86-2.05)	.52	—	

HR indicates hazard ratio; CI, confidence interval; WBC, white blood cell; —, not applicable; and TBI, total body irradiation.

In conclusion, comparable survival rates were observed between adult Ph⁻ ALL patients who underwent related and unrelated allo-SCTs in CR1, although relapse rates, incidences of NRM, and risk factors for transplantation outcomes were different between them. Better outcomes could be achieved by performing allo-SCT at an appropriate timing and HLA compatibility according to donor type.

Table 5. Causes of death among patients transplanted in CR1, according to donor type

	Related (n = 310)		Unrelated (n = 331)		
	n	%	n	%	P
Relapse	44	44	32	26	.01
Infection	12	12	23	19	.20
Organ failure	12	12	17	14	.83
GVHD	9	8.9	16	13	.40
Interstitial pneumonia	5	5.0	15	12	.06
Hemorrhage	3	3.0	6	5.0	.52
Graft failure	2	2.0	3	2.5	1.0
ARDS	1	1.0	3	2.5	.63
Other	8	7.9	6	5.0	.42
Unknown	5	5.0	0	0.0	.02
Total	101	100	121	100	

ARDS indicates acute respiratory distress syndrome.

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Authorship

Contribution: S.N., Y.I., and K.M. designed the research and wrote the manuscript; S.N. and Y.I. performed the statistical analysis and interpreted the data; H.S., M. Kurokawa, H.I., H.O., T.F., Y.O., N.K., M. Kasai., T.M., K.I., T.Y., M.O., and

K.M. provided the patient data; and K.K., Y.M., R.S., and Y.A. collected the patient data.

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ORIGINAL ARTICLE

Peripheral blood stem cell versus bone marrow transplantation from HLA-identical sibling donors in patients with leukemia: a propensity score-based comparison from the Japan Society for Hematopoietic Stem Cell Transplantation registry

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Abstract We retrospectively analyzed the results of 707 adult patients who underwent myeloablative peripheral blood stem cell transplantation (PBSCT) ($n = 365$) and myeloablative bone marrow transplantation (BMT) ($n = 342$) for leukemia from HLA-identical sibling donors between 2000 and 2005 using the propensity score method. The results were obtained from the Japan Society for Hematopoietic Cell Transplantation registry. Multivariate Cox analysis showed that PBSCT was associated with lower overall survival (OS) in standard-risk patients [adjusted hazard ratio (aHR) = 1.83; 95% confidence interval (CI) 1.04–3.23; $P = 0.036$], but not in high-risk patients (aHR = 1.11; 95% CI 0.76–1.61; $P = 0.599$). Hematopoietic recovery was significantly faster after

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PBSCT. The risk of acquiring grade III–IV acute graft-versus-host disease (GVHD) (aHR = 2.23; $P = 0.040$) and extensive chronic GVHD (aHR = 1.93; $P = 0.001$) were significantly higher after PBSCT. PBSCT was associated with higher non-relapse mortality in standard-risk patients (aHR = 2.30; 95% CI 1.08–4.88; $P = 0.030$), but not in high-risk patients (aHR = 1.29; 95% CI 0.65–2.54; $P = 0.468$). Relapse after transplantation did not differ between PBSCT and BMT either in standard-risk group or in high-risk group (aHR = 1.17; 95% CI 0.55–2.52; $P = 0.684$ and aHR = 0.81; 95% CI 0.52–1.28; $P = 0.370$, respectively). In this retrospective analysis, OS was significantly lower after PBSCT in standard-risk patients, but not in high-risk patients. PBSCT was associated with significant risks of grade III–IV acute GVHD and extensive chronic GVHD.

Keywords Bone marrow transplantation · Peripheral blood stem cell transplantation · Allogeneic · Graft-versus-host disease

1 Introduction

During the past decade, allogeneic peripheral blood stem cell transplantation (allo-PBSCT) has been increasingly used as an alternative to allogeneic bone marrow transplantation (allo-BMT) [1]. Furthermore, allo-PBSCT is associated with rapid hematopoietic recovery. Several prospective randomized controlled trials conducted in Western countries have shown an increased incidence of chronic graft-versus-host disease (GVHD) [2–11]. Nevertheless, there is still substantial controversy regarding survival, acute GVHD, non-relapse mortality (NRM), and relapse [12–14].

Ethnicity has been reported to affect the incidence and severity of GVHD [15]. Japanese patients undergoing allogeneic hematopoietic stem cell transplantation (HSCT) were found to have a lower incidence of acute GVHD than those from Western patients [16, 17]. Therefore, the outcome of allo-PBSCT compared with that of allo-BMT may differ according to the ethnic background.

Using the propensity score method, we retrospectively analyzed the clinical outcomes of 707 adult Japanese leukemia patients who received allogeneic HSCT with myeloablative conditioning from HLA-identical sibling donors. These data were obtained from the Japan Society for Hematopoietic Cell Transplantation (JSHCT) registry. A propensity scoring system was devised to estimate the effects of treatments by comparing outcomes of those subjects who were not randomly assigned to experimental or control groups in an observational study [18]. A randomized control trial is superior in eliminating the confounding factors of known and unknown covariates by random treatment assignment. The propensity score expresses the likelihood of being assigned to experimental or control treatments, and is calculated using logistic regression models, including variables measured prior to treatment as much as possible. Considering the propensity score in this analysis, we expected that a hypothetical evaluation of an experimental trial in an observational study would give results similar to those of an evaluation in a randomized controlled trial.

2 Patients and methods

2.1 Study population

Using a standardized reporting form, JSHCT collects data on individual transplant patients from each transplant center, and follow-up reports are submitted annually after transplantation. A total of 1,426 patients, who underwent allogeneic HSCT between 2000 and 2005 for acute myelogenous leukemia (AML), acute lymphoblastic leukemia (ALL), and chronic myelogenous leukemia (CML), have been reported to JSHCT. Patients were excluded from the study if their data were incomplete ($n = 205$), if they received a non-myeloablative or reduced-intensity conditioning regimen ($n = 223$), if they received grafts from other than HLA-identical siblings ($n = 217$), if they were less than 18 years of age ($n = 38$), if they had a previous history of HSCT ($n = 10$), and if they had non-allo-PBSCT or non-allo-BMT ($n = 16$). In Japan, most

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allo-HSCT patients have received granulocyte-colony stimulating factor (G-CSF) post-transplant [19]. The August 2006 data of the remaining 707 patients were analyzed. This study was approved by the Data Management Committee for the Nationwide Survey of JSHCT.

2.2 Definitions

Risk status at transplantation was categorized as either standard or high. Standard-risk diseases included acute leukemia in first complete remission (CR) and CML in first chronic phase (CP). Other disease status was categorized as high-risk disease [11]. The day of neutrophil engraftment was defined as the first of three consecutive days with an absolute neutrophil count (ANC) of more than 0.5×10^9/L. The day of platelet engraftment was defined as the first of seven consecutive days with a platelet count of more than 20×10^9/L without platelet transfusion. Acute GVHD was graded according to the standard criteria [20]. All patients who had no evidence of graft failure and survived beyond day 28 were considered to be evaluable for acute GVHD. GVHD persisting beyond day 100 and de novo GVHD occurring after day 100 were classified as chronic GVHD. The incidence of chronic GVHD was calculated in patients followed for more than 100 days, and the disease was classified as none, limited, or extensive [21]. Overall survival (OS) was defined as the duration of survival between transplantation and either death or the last follow-up.

Relapse was defined as disease progression with censored NRM. NRM included all causes of death other than relapse occurring at any time after transplantation. All deaths were considered in the estimation of OS.

2.2.1 Endpoints

The primary endpoint of comparison was OS. Secondary endpoints were hematopoietic recovery, acute GVHD (grade II–IV and III–IV), chronic GVHD (overall and extensive), NRM, and relapse.

2.2.2 Propensity score calculation

We calculated the propensity score using the pscore command in STATA version 10.1. (STATA, College Station, TX, USA) [22]. Factors included in the propensity score were as follows: age at HSCT in categories (<40, 40–49, and 50+) as an ordinal variable; sex (male/female) as an indicator variable; year of transplantation as a continuous variable; performance status at transplantation as an ordinal variable; risk status (CR1/CP1, CR2/CP2, or more advanced) as an indicator variable; a cumulative number of HSCT from related donors at an institution between 2000 and 2005 (1: 1–4, 62 institutions; 2: 5–11, 58 institutions; 3: 12 or more, 52 institutions) as an ordinal variable; and the percentage of allo-PBSCT out of total HSCT from HLA-identical siblings in tertile (1: <56%, 59 institutions; 2: 56–90%, 56 institutions; 3: 91% or more, 57 institutions) as an ordinal variable. We utilized as many variables as possible in the propensity score to evaluate the effects of known and unknown factors on the choice of treatment. After calculating the propensity score, the subjects were divided into four groups according to quartile. The numbers of subjects in quartiles 1–4 (allo-PBSCT/allo-BMT) were 23/154, 58/120, 126/50, and 158/18, respectively.

2.2.3 Statistical analysis

Patient characteristics and therapeutic outcomes were compared between allo-PBSCT and allo-BMT groups. OS was assessed using the Kaplan–Meier product limit method [23, 24]. Cumulative incidences of acute GVHD, chronic GVHD, NRM, and relapse were evaluated as 1 − (Kaplan–Meier estimate) instead of applying methods considering competing risks [25, 26] to maintain statistical consistency between logrank tests and methods of cumulative incidence estimation. Allo-PBSCT and allo-BMT groups were compared using the propensity score in quartiles [1–4], a stratified logrank test, and a stratified Cox proportional hazards model. Diagnosis (AML, ALL, and CML) and quartile of the propensity score were stratification factors. Confounders considered in the Cox proportional hazards model were as follows: year of diagnosis as a continuous variable; year of transplantation as a continuous variable; age at transplantation as a continuous variable; sex (male/female); sex matching (match/male to female/female to male/unknown); performance status (0, 1, 2, 3–4, and unknown); risk status (standard/high); GVHD prophylaxis [cyclosporin (CsA) + methotrexate (MTX), tacrolimus (TAC) + MTX, and others]; and conditioning regimen [total body irradiation (TBI)-containing regimen, busulfan and cyclophosphamide (BU/CY), and others]. All analyses were performed using STATA version 10.1, and P values less than 0.05 were considered statistically significant.

3 Results

3.1 Patient characteristics

The characteristics of patients are summarized in Table 1. The number of patients who underwent allo-PBSCT was 365, and that who underwent allo-BMT was 342. The median age at HSCT was 39 years (range 18–64 years) in the allo-PBSCT group and 39 years (range 18–59 years) in the allo-BMT group. The allo-PBSCT group included significantly more male patients from female donors than

Table 1 Characteristics of patients

	PBSCT *n* (%)	BMT *n* (%)	*P* value (Mann–Whitney test)
No. of patients	365	342	
Median patients age, years (range)	39, 18–64	39, 18–59	0.962
Patients sex (male/female)	210/155	189/153	0.543
Sex matching			
Matched	176 (48.2)	185 (54.1)	
Male to female	70 (19.2)	78 (22.8)	
Female to male	106 (29.0)	71 (20.8)	
Unknown	13 (3.6)	8 (2.3)	0.043
Risk group			
Standard-risk	149 (40.8)	202 (59.1)	
High-risk	216 (59.2)	140 (40.9)	<0.001
Diagnosis			
Standard-risk			
AML	58 (38.9)	76 (37.6)	
ALL	46 (30.9)	51 (25.2)	
CML	45 (30.2)	75 (37.2)	0.322
High-risk			
AML	128 (59.3)	75 (53.6)	
ALL	58 (26.9)	28 (20.0)	
CML	30 (13.8)	37 (26.4)	0.026
Performance status			
0	185 (50.7)	138 (40.4)	
1	73 (20.0)	55 (16.1)	
2	24 (6.6)	16 (4.7)	
3 or 4	12 (3.3)	2 (0.6)	
Unknown	71 (19.5)	131 (38.3)	<0.001
Conditioning regimen			
TBI-based	225 (61.6)	205 (59.9)	
Bu/CY	110 (30.1)	118 (34.5)	
Others	30 (8.3)	19 (5.6)	0.23
GVHD prophylaxis			
CsA + MTX	308 (84.4)	300 (87.7)	
TAC + MTX	12 (3.3)	14 (4.1)	
Others	45 (12.3)	28 (8.2)	0.176

Standard-risk diseases: acute leukemia in first complete remission and chronic myelogenous leukemia in first chronic phase; other disease status was categorized as high-risk diseases

PBSCT peripheral blood stem cell transplantation, *BMT* bone marrow transplantation, *AML* acute myelogenous leukemia, *ALL* acute lymphoblastic leukemia, *CML* chronic myelogenous leukemia, *TBI* total body irradiation, *Bu* busulfan, *CY* cyclophosphamide, *GVHD* graft-versus-host disease, *CsA* cyclosporin, *MTX* methotrexate, *TAC* tacrolimus

the allo-BMT group (Mann–Whitney test, $P = 0.043$). AML, ALL, and CML were diagnosed in 337, 183, and 187 patients, respectively. The allo-PBSCT group included significantly more high-risk patients than the allo-BMT group ($P < 0.001$). Among the high-risk patients, the allo-BMT group had significantly more CML patients than the allo-PBSCT group ($P = 0.026$). Conditioning regimen and GVHD prophylaxis were performed according to the protocol of each institution, and there were no differences between the two groups. The most frequently used conditioning regimens were BU/CY (busulfan 1 mg/kg × 4/day × 4 days with cyclophosphamide 60 mg/kg/day × 2 days) and CY/TBI (cyclophosphamide 60 mg/kg/day × 2 days with total body irradiation 10–12 Gy). CsA plus MTX was used most frequently for GVHD prophylaxis. Median follow-up period for the surviving patients at the time of analysis was 33 months (1.8–55 months) in the PBSCT group and 31 months (1–53 months) in the BMT group.

3.2 Primary endpoint

3.2.1 Overall survival

Three-year OS in standard-risk patients was 68% [95% confidence interval (CI) 59–75] after allo-PBSCT and 77%

(95% CI 70–82) after allo-BMT (by disease and quartile in the propensity-score stratified logrank test; $P = 0.023$). Three-year OS in high-risk patients after allo-PBSCT and allo-BMT was 38% (95% CI 31–45) and 54% (95% CI 44–62), respectively ($P = 0.587$) (Fig. 1). Multivariate Cox analysis showed that allo-PBSCT was a significant factor for lower OS in the population with standard-risk [adjusted hazard ratio (aHR) = 1.83; 95% CI 1.04–3.23; $P = 0.036$], but not that with high-risk (aHR = 1.11; 95% CI 0.76–1.61; $P = 0.599$).

3.3 Secondary endpoints

3.3.1 Hematopoietic recovery

Engraftment occurred in all patients receiving allo-PBSCT and allo-BMT (allo-PBSCT, $n = 324$; allo-BMT, $n = 305$) surviving for more than 28 days. Allo-PBSCT patients showed significantly faster neutrophil and platelet recovery compared with allo-BMT patients. The median time of recovery to ANC $> 0.5 \times 10^9$/L was 14 days for the allo-PBSCT group and 16 days for the allo-BMT group, respectively (stratified logrank test, $P < 0.0001$). The median time of recovery to a platelet count $>20 \times 10^9$/L was 15 days for the allo-PBSCT group and 21 days for the allo-BMT group, respectively ($P < 0.0001$). In the multivariate Cox analysis, allo-PBSCT was a significant factor for faster neutrophil (aHR = 0.57; 95% CI 0.45–0.71; $P < 0.001$) and platelet (aHR = 0.56; 95% CI 0.44–0.71; $P < 0.001$) recovery compared with allo-BMT.

3.3.2 Acute GVHD

The cumulative incidence of grade II–IV acute GVHD was 31% (95% CI 27–35) in all patients, whereas that in allo-PBSCT and allo-BMT groups was 35% (95% CI 30–41) and 26% (95% CI 22–32) (stratified logrank test, $P = 0.221$), respectively. The aHR for grade II–IV acute GVHD after allo-PBSCT was 1.25 (95% CI 0.85–1.84; $P = 0.260$) by multivariate Cox analysis. The cumulative incidence of grade III–IV acute GVHD was 14% (95% CI 10–18) and 5.4% (95% CI 3.3–8.8) in the allo-PBSCT and allo-BMT groups, respectively ($P = 0.021$). Multivariate Cox analysis showed that allo-PBSCT was a significant factor for the development of grade III–IV acute GVHD (aHR = 2.23; 95% CI 1.04–4.78; $P = 0.040$; Fig. 2).

3.3.3 Chronic GVHD

The risk of chronic GVHD in the first year after transplantation was significantly higher after allo-PBSCT than after allo-BMT (cumulative incidence at 1 year, 51%; 95% CI 44–58 after allo-PBSCT vs. 34%; 95% CI 28–41 after allo-BMT; $P = 0.0005$ with stratified logrank test). The extensive form of chronic GVHD was more prevalent in the allo-PBSCT group than in the allo-BMT group (26%; 95% CI 21–33 with allo-PBSCT and 15%; 95% CI 11–20 with allo-BMT; $P = 0.0017$). Multivariate Cox analysis showed that allo-PBSCT was a significant factor for the development of extensive chronic GVHD (aHR = 1.93; 95% CI 1.32–2.84; $P = 0.001$; Fig. 3).

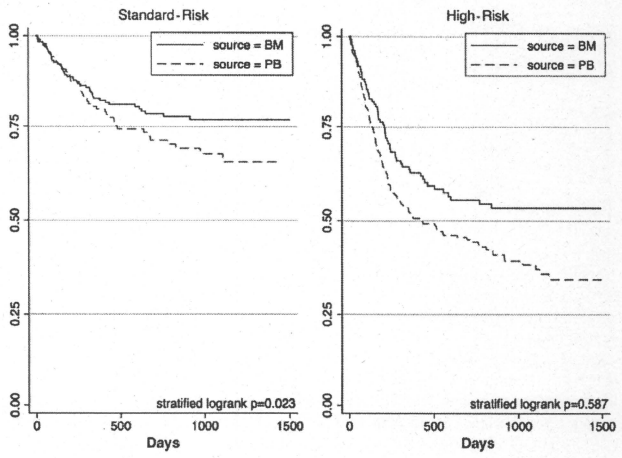

Fig. 1 Probabilities of overall survival after peripheral blood stem cell transplantation compared with bone marrow transplantation. Standard-risk diseases included acute leukemia in first complete remission and chronic myelogenous leukemia in first chronic phase. Other diseases were categorized as high-risk diseases

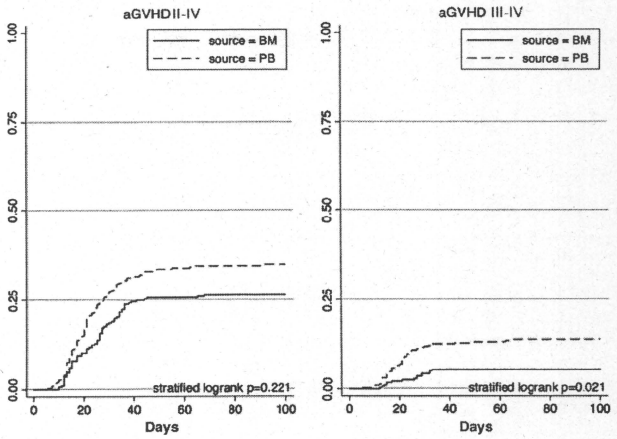

Fig. 2 Probabilities for grade II–IV and III–IV acute graft-versus-host disease (GVHD) after peripheral blood stem cell transplantation compared with bone marrow transplantation

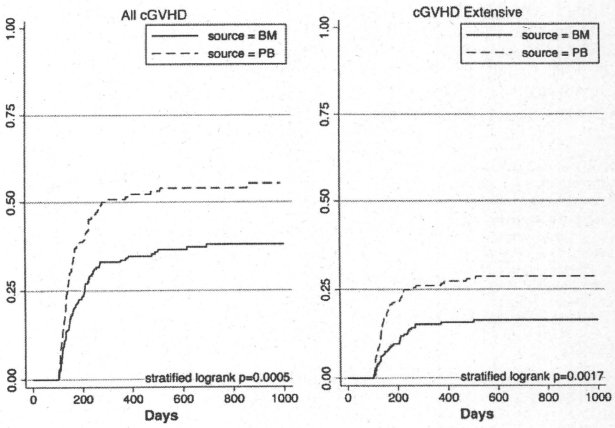

Fig. 3 Probabilities for chronic GVHD and extensive chronic GVHD after peripheral blood stem cell transplantation compared with bone marrow transplantation

3.3.4 Non-relapse mortality

The cumulative incidence of NRM for the standard-risk group at day 100 was 4.7% (95% CI 2.3–9.7) after allo-PBSCT and 6.0% (95% CI 3.4–10.2) after allo-BMT, and that at 1 year was 14.2% (95% CI 9.4–21.1) after allo-PBSCT and 11.2% (95% CI 8.0–17.2) after allo-BMT (stratified logrank test, $P = 0.047$). The cumulative incidence of NRM for the high-risk group at day 100 was 11.2% (95% CI 7.6–16.4) after allo-PBSCT and 8.9% (95% CI 5.1–15.1) after allo-BMT, and that at 1 year was 24.4% (95% CI 18.7–31.4) after allo-PBSCT and 14.7% (95% CI 9.6–22.2) after allo-BMT (stratified logrank test, $P = 0.221$) (Fig. 4).

Multivariate Cox analysis showed that allo-PBSCT was a significant factor for higher NRM in the standard-risk (aHR = 2.30; 95% CI 1.08–4.88; $P = 0.030$), but not in the high-risk (aHR = 1.29; 95% CI 0.65–2.54; $P = 0.468$).

3.3.5 Relapse

The cumulative incidence of relapse at 1 year for the standard-risk group was similar for allo-PBSCT (13.8%; 95% CI 8.9–21.0) and allo-BMT (9.7%; 95% CI 6.1–15.2) ($P = 0.518$ by stratified logrank test). Similarly, in the high-risk group the incidence was 32.4% (95% CI 25.6–40.3) for allo-PBSCT and 31.5% (95% CI 23.7–41.1) for allo-BMT ($P = 0.200$) (Fig. 5).

Multivariate Cox analysis showed no significant difference in the risk of relapse after allo-PBSCT and allo-BMT either in the standard-risk group or in the high-risk group (aHR = 1.17; 95% CI 0.55–2.52; $P = 0.684$ and aHR = 0.81; 95% CI 0.52–1.28; $P = 0.370$, respectively).

4 Discussion

In the present study, we analyzed results for 707 patients who underwent myeloablative HSCT for leukemia from HLA-identical sibling donors between 2000 and 2005. These data were obtained from the JSHCT registry. Health insurance coverage of allo-PBSCT was approved in Japan in 2000, and since then the number of allo-PBSCTs rapidly increased and exceeded the number of allo-BMTs between 2000 and 2003. Subsequently, the number of allo-PBSCTs decreased, and the numbers of allo-PBSCTs and allo-BMTs became equivalent in 2005. Thus, this analysis indicates the rather immature status of allo-PBSCT in Japan.

The Stem Cell Trialists' Collaborative Group [27] reported an individual patient data meta-analysis of nine randomized trials by comparing outcomes of allo-PBSCT versus allo-BMT from HLA-matched related donors for the treatment of hematologic malignancies. Allo-PBSCT was associated with a higher probability of 5-year OS in the subset analysis of patients with late disease due to decreased relapse. International Bone Marrow Transplant registry/European Group for Blood and Marrow Transplantation (IBMTR/EBMT) registry data of 398 adult allo-BMT and 208 allo-PBSCT patients with leukemia were analyzed using information on 6 or more years of follow-up [28]. OS in patients with early and advanced leukemia did not differ significantly between the two groups. The IBMTR report comparing outcomes after allo-PBSCT and allo-BMT for acute leukemia in children and adolescents showed that OS was lower after allo-PBSCT [29]. These controversial data indicate that the difference in stem cell source can affect OS depending on the underlying disease, disease status, and the patients' age.

In our study, OS was lower after allo-PBSCT than after allo-BMT in the standard-risk patients, but not in the high-risk patients. Considering the difference in stem cell source, factors affecting OS include hematopoietic and

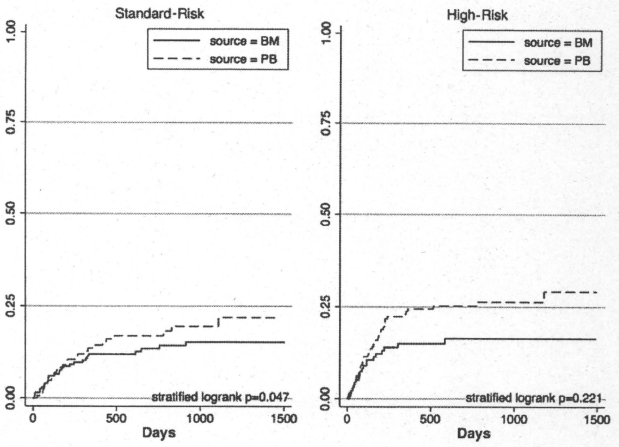

Fig. 4 Cumulative incidences of non-relapse mortality (NRM) after peripheral blood stem cell transplantation compared with bone marrow transplantation. Standard-risk diseases included acute leukemia in first complete remission and chronic myelogenous leukemia in first chronic phase. Other diseases were categorized as high-risk diseases

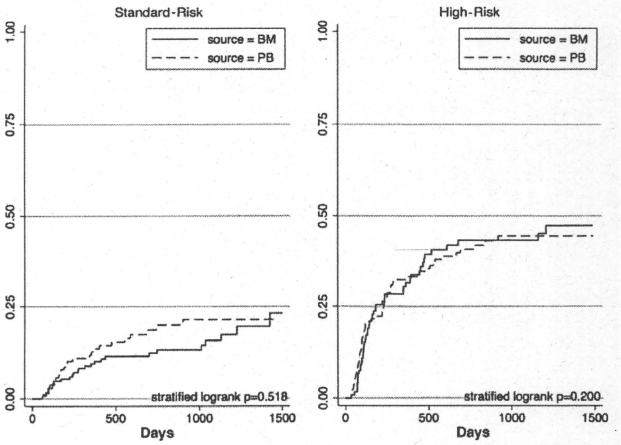

Fig. 5 Cumulative incidences of relapse after peripheral blood stem cell transplantation compared with bone marrow transplantation. Standard-risk diseases included acute leukemia in first complete remission and chronic myelogenous leukemia in first chronic phase. Other diseases were categorized as high-risk diseases

immune recovery, acute and chronic GVHD, and graft-versus-leukemia (GVL) effect or relapse [30].

In our analysis, allo-PBSCT was associated with more rapid hematopoietic recovery than allo-BMT as has been shown in most previous studies [4, 5, 11, 31]. Most randomized trials demonstrated that neutrophil recovery generally occurs 5–7 days earlier after allo-PBSCT compared with allo-BMT without G-CSF post-transplant [27, 32]. The EBMT study reported by Schmitz et al. [5] showed that neutrophil recovery was achieved 3 days earlier after allo-PBSCT than after allo-BMT with G-CSF post-transplant, and transplantation-related mortality did not differ between allo-PBSCT and allo-BMT groups. In Japan, most allo-HSCT patients receive G-CSF post-transplant, and in our study neutrophil recovery was observed 2 days earlier after allo-PBSCT than after allo-BMT. Accordingly, infectious complications may not decrease after allo-PBSCT compared to allo-BMT.

With regard to acute GVHD, the meta-analysis showed that allo-PBSCT was associated with a significant increase in the development of grade III–IV acute GVHD, but not grade II–IV acute GVHD [27]. In the present analysis, allo-PBSCT was also a significant factor in the incidence of grade III–IV acute GVHD. The increased incidence of grade III–IV acute GVHD in allo-PBSCT would have a negative effect on OS [33].

Extensive chronic GVHD was more frequent after allo-PBSCT than after allo-BMT in our study. This finding is in line with those of previous reports [5, 9, 11, 19, 31, 34].

In our analysis, NRM was higher after allo-PBSCT in the standard-risk patients, but not in the high-risk patients. The higher NRM after allo-PBSCT in the standard-risk group was likely due to increased grade III–IV acute GVHD and extensive chronic GVHD. Increased NRM after allo-PBSCT has been reported from children and adolescents suffering with acute leukemia [29]. A higher risk of mortality due to acute and chronic GVHD may counteract any benefit of more rapid hematopoietic recovery in the early transplant period.

In the allo-BMT setting, the development of both acute and chronic GVHD is associated with decreased relapse of leukemia, whereas the effect of GVHD on OS appears to be different depending on the study population [33, 35, 36]. The meta-analysis showed that allo-PBSCT was associated with a significant decrease in relapse in both early and late-stage disease patients [27]. On the contrary, increased extensive chronic GVHD in the allo-PBSCT group did not lead to a decrease in relapse in our analysis. We do not have a good explanation for this, but a similar observation was reported from the IBMTR/EBMT [28] registry data of adult patients with leukemia and the IBMTR [29] study in children and adolescents with acute leukemia. The advantage in term of the GVL effect with the cost of increased GVHD after allo-PBSCT relative to after allo-BMT remains controversial [27–29]. The allogeneic GVL effect varies from one disease to another, with the stage of the disease, and with donor histocompatibility. The GVL effect is believed to act while the leukemic burden is relatively

low [37]. Thus, to investigate the relationship between GVHD and relapse, subgroups differing in underlying disease and disease status would be needed.

We used the propensity score method to minimize selection bias. However, retrospective analysis has limitations. We could not exclude the possibility of unidentified confounding variables affecting the transplant outcomes and the inability to adjust the data for unknown or unmeasured factors. For example, we did not have data regarding pre-transplant infectious complications. Since allo-PBSCT is associated with more rapid hematopoietic recovery than allo-BMT, patients with serious infectious problems may have a tendency to undergo allo-PBSCT rather than allo-BMT. In this analysis, standard-risk diseases included acute leukemia in first CR and CML in first CP, while high-risk diseases included other diseases [11]. However, even in first CR acute leukemia patients, cytogenetic and molecular markers affect the prognosis with respect to survival in the allo-HSCT setting [38, 39]. We cannot deny the possibility that higher-risk patients in first CR tended to undergo allo-PBSCT. Thus, the results presented here should be interpreted with caution. It is also important to realize that our analysis was based on matched sibling myeloablative HSCT not on non-myeloablative HSCT. However, contrary to the result of the meta-analysis [27], multivariate Cox analysis showed that the allo-PBSCT group was associated with a lower OS in the populations with standard-risk. Prospective randomized trials are necessary to elucidate the advantages and disadvantages of allo-PBSCT in comparison with allo-BMT from HLA-identical sibling donors for the treatment of adult Japanese patients with leukemia.

Conflict of interest statement The authors declare no financial conflict of interest.

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Brief communication

Prevalence and clinical characteristics of N-terminally truncated WT1 expression in acute myeloid leukemia

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ABSTRACT

Overexpression of the Wilms' tumor gene 1 (*WT1*) was observed in most leukemia cells. In addition to four major isoforms of WT1, an N-terminally truncated isoforms (sWT1) has been identified. We separately quantified the transcript levels of sWT1 and full-length WT1 (fWT1) in 237 patients with acute myeloid leukemia (AML). sWT1 expression was observed in 45 of 237 (19.0%) AML patients, particularly in acute promyelocytic leukemia (59.3%). Although sWT1 expression was not associated with other genetic mutations and prognosis, fWT1 expression level in sWT1-expressing AML was significantly higher than that in un-expressing AML. These results suggested the possible cooperation of sWT1 and fWT1 in the pathophysiology of AML, while further analysis is required.

1. Introduction

The Wilms' tumor gene 1 (*WT1*) plays an important role in the normal development of several organs in vertebrates, especially the urogenital system. Overexpression of the *WT1* gene has been reported in a variety of cancers, as well as leukemia [1]. The WT1 has various isoforms, due to its alternative splicing of exon 5 consisted of 17 amino acids (17AA) and a stretch of three amino acids, lysine, threonine and serine (KTS) at the 3′ end of exon 9 [2]. These two splicing events give rise four major different WT1 isoforms: 17AA+/KTS+, 17AA+/KTS−, 17AA−/KTS+ and 17AA−/KTS−, and their different biological roles for cell differentiation and proliferation have been reported. In addition to the four major isoforms, it has been reported that other isoforms translated from upstream or downstream initiations result in larger or smaller WT1 protein, respectively [3,4]. N-terminally truncated isoforms resulting from translation initiation at a downstream AUG codon located in a cryptic exon within intron 1 produces a ~35–37 kDa smaller WT1 protein (sWT1) [5,6]. The sWT1 transcript is expressed in human testis and fetal kidney, but not in the adrenal gland where the full-length WT1 (fWT1) transcript is expressed. Although DNA-binding capacity of sWT1 is the same as that of fWT1, sWT1 reportedly activates the promoter activity of *cycline E* and the *insulin-like growth factor 1 receptor* (*IGF-1R*) genes, which are normally repressed by fWT1. Furthermore, sWT1 transcript was predominantly observed in 12 of the 52 (23.1%) human leukemia samples [6]. These results collectively suggested that sWT1 might exhibit oncogenic properties and be involved in the pathophysiology of leukemia, while the precise role of sWT1 in leukemogenesis and the characteristics of sWT1-expressing leukemia cells have not been fully elucidated.

In this study, we separately quantified the transcript levels of sWT1 and fWT1 in bone marrow (BM) cells from 237 patients with acute myeloid leukemia (AML), and analyzed the relationship between the expression levels of both transcripts and the clinical characteristics of sWT1-expressing AML in consideration of other genetic alterations.

2. Materials and methods

2.1. Patients and samples

The diagnosis of AML was based on the morphology, histopathology, the expression of leukocyte differentiation antigens and/or the French-American-British (FAB) classification. For the normal control, four BM, four peripheral blood (PB) and five cord blood (CB) mononuclear cells (MNCs) were used. We obtained informed consent from all patients and volunteers to use their samples for banking and molecular analysis, and approval was obtained from the ethics committee of Nagoya University School of Medicine. CB was collected after full-term deliveries with informed consent approved by the Review Board of Tokai Cord Blood Bank. Total RNAs of human testis, fetal kidney and adult kidney were purchased from Clontech (Mountain View, CA).

2.2. Quantification of sWT1 and fWT1 transcripts

Extraction of total RNA from BM AML cells and synthesis of cDNA were performed by standard methods. The individual expression level of fWT1 and

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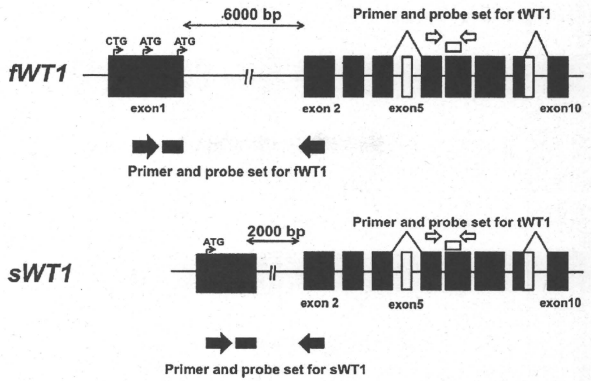

Fig. 1. Schematic representation of the full-length WT1 (fWT1) and N-terminally truncated WT1 (sWT1) transcripts. Primer and probe sets for quantitating fWT1, sWT1 and the total amount of WT1 are indicated.

sWT1 transcript was quantitated using a real-time fluorescence detection method on an ABI prism7000 sequence detection system (Applied Biosystems, Foster City, CA). The primer and probe sequences for real-time PCR of fWT1 and sWT1 were as follows: sense primer, antisense primer and Taqman probe for fWT1 were 5′-CCAGGCCAGGATGTTTCCTAAC-3′ at exon 1, 5′-GGATCCTCATGCTTGAATGAGTGG-3′ at exon 2 and 5′-AGAGCCAGCCCGCTATTCGCAAT-3′ at exon 1, respectively; those for sWT1 were 5′-AGGGCTCTGAGGATTGTGCAGT-3′ at intron 1, 5′-GGATCCTCATGCTTGAATGAGTGG-3′ at exon 2 and 5′-TCCTGGGCTCAGGAATGGAGAAG-3′ at intron 1, respectively (Fig. 1). To quantify the total amount of WT1 transcript, sense primer 5′-GATAACCACACAACGCCCATC-3′ at exon 6, antisense primer 5′-GACACGTCGCACATCCTGAAT-3′ at exon 7 and Taqman probe 5′-CCAATACAGAATACACACGCACGGTCT-3′ at exon 6 were used as previously reported. The *GAPDH*, served as a control of cDNA quality. Each gene expression level was calculated as previously described [7]. Using serially diluted samples, we determined the thresholds of both fWT1 and sWT1 expressions as 50 copy/μgRNA.

2.3. Cytogenetics and mutation analysis

Cytogenetic G-banding analysis was performed with standard methods. Mutations of *FLT3*, *KIT*, *NPM1*, *CEBPA*, *NRAS*, *TP53* and *WT1* genes were analyzed as previously reported [8].

3. Results and discussion

Consistent with the previous report [6], fWT1 expression was strong in the testis (1,100,000 copy/μgRNA), fetal kidney (650,000 copy/μgRNA) and adult kidney (60,000 copy/μgRNA), though it was weak in normal MNCs of BM (135–916 copy/μgRNA) and CB (261–2100 copy/μgRNA). In contrast, sWT1 expression was observed in the testis (350,000 copy/μgRNA) and fetal kidney (1100 copy/μgRNA), but not in the adult kidney and MNCs of PB, BM and CB (Fig. 2A). These results confirmed that our quantitation system was well established and that sWT1 expression was more restricted to fetal organs than fWT1 expression.

Although fWT1 expression was observed in most AML cases (224/237, 94.5%), sWT1 expression was observed only in 45 cases (19.0%) (Table 1). sWT1 expression level varied from 50 to 20,375 copy/μgRNA, while its expression level was lower than the fWT1 expression level in each case (Fig. 2B). Furthermore, the median fWT1 expression level in sWT1-expressing cases was significantly higher than in sWT1-non-expressing cases (104,991 and 20,417 copy/μgRNA, respectively, $p < 0.0001$ by the Mann–Whitney *U*-test) (Fig. 2C). A strong correlation between the total amount of WT1 (tWT1) expression level, which was quantified by the primer and probe set corresponding to exon 6 and 7, and fWT1 expression levels was observed ($r = 0.947$), but not between sWT1 and tWT1 transcripts ($r = 0.028$). These results collectively indicated that sWT1 is not a major isoform in human leukemia cells, while sWT1 expression might be regulated by the fWT1 expression level.

Table 1
Association of sWT1 expression with clinical and genetic characteristics in AML.

	Total	sWT1 expression	
		Positive	Negative
Number	237	45 (19.0%)	192 (81.0%)
Age (y) (range)	48.5 (16–81)	46 (16–81)	49 (16–77)
WBC × 10^9/L (range)	16.3 (0.6–372)	6.9 (0.6–372)	19.1 (0.7–315)
Cytogenetics			
t(8;21)	28	3 (10.7%)	25 (89.3%)
inv(16)	6	2 (33.3%)	4 (66.7%)
t(15;17)	27	16 (59.3%)	11 (40.7%)
11q23 abn.	5	2 (40.0%)	3 (60.0%)
Chr 5/7 abn.	8	3 (37.5%)	5 (62.5%)
t(9;22)	3	0 (0%)	3 (100%)
Complex	14	3 (21.4%)	11 (78.6%)
Other abn.	35	3 (8.6%)	32 (91.4%)
Normal	82	9 (11.0%)	73 (89.0%)
Unknown	29	4 (13.8%)	25 (86.2%)
FLT3			
Wild	173	27 (15.6%)	146 (84.4%)
Mutation	59	16 (27.1%)	43 (72.9%)
NPM1			
Wild	175	27 (15.4%)	148 (84.6%)
Mutation	45	10 (22.2%)	35 (77.8%)
KIT			
Wild	91	24 (26.4%)	67 (73.6%)
Mutation	4	0 (0%)	4 (100%)
CEBPA			
Wild	78	22 (28.2%)	56 (71.8%)
Mutation	11	1 (9.1%)	10 (90.9%)
TP53			
Wild	203	34 (16.7%)	169 (83.3%)
Mutation	17	3 (17.6%)	14 (82.4%)
NRAS			
Wild	201	33 (16.4%)	168 (83.6%)
Mutation	25	5 (20.0%)	20 (80.0%)
WT1			
Wild	150	28 (18.7%)	122 (81.3%)
Mutation	4	2 (50.0%)	2 (50.0%)

sWT1 expression was significantly associated with the low WBC count ($p = 0.0083$ by the Mann–Whitney U-test). In addition, sWT1 expression was preferentially observed in the cases with *t*(15;17), while it was not associated with mutations of FLT3, NPM1, KIT, NRAS, CEBPA, WT1, TP53 and WT1 genes.

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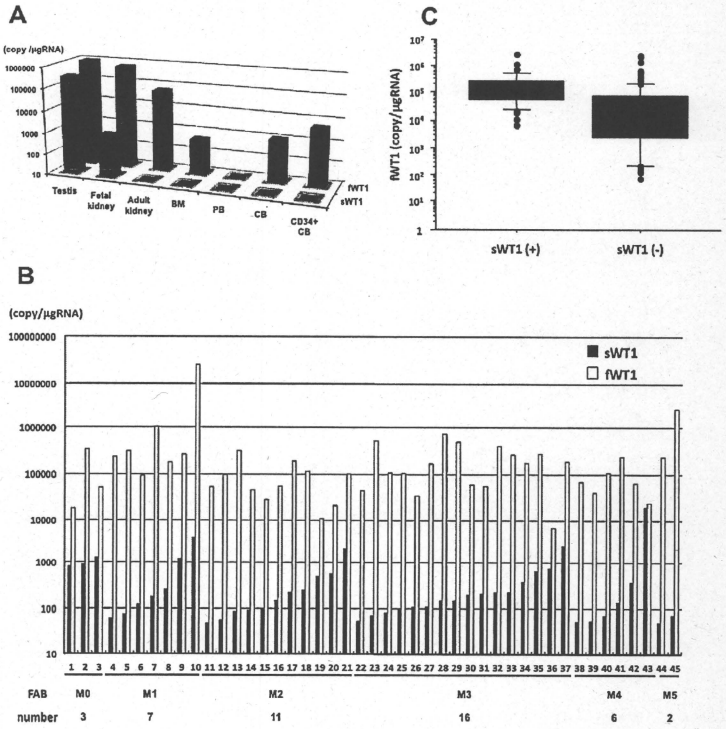

Fig. 2. (A) Expression levels of sWT1 and fWT1 transcripts in normal samples. sWT1 was expressed in the testis and fetal kidney, but not in the adult kidney and normal hematopoietic cells. (B) Comparison of the expression levels between sWT1 and fWT1 transcripts in 45 sWT1-expressing AML. The sWT1 expression level varied from 50 to 20,375 copy/μgRNA, while there was no predominantly sWT1 expressing case. (C) fWT1 expression level in sWT1-expressing cases was significantly higher than in sWT1-non-expressing cases ($p < 0.0001$).

Hossain et al. first reported that sWT1 expression was observed in 20 of 52 (38.5%) acute leukemia cases, including 12 (23.1%) cases, which predominantly express a single sWT1 isoform; however, the expression level of sWT1 transcript in our study was apparently lower than that of the previous report and there was no predominantly sWT1 expressing case [6]. Although our quantitating system could sensitively determine the sWT1 transcript in normal testis and fetal kidney, different primer and probe sequences for quanti-

Table 2
Expression of sWT1 and fWT1 transcripts in AML according to the FAB type.

FAB type	Total	sWT1 expression		fWT1 (copy/μgRNA)	
		Positive	Negative	Median	Range
M0	11	3 (29.3%)	8 (70.7%)	52,357	0–331,409
M1	39	7 (17.9%)	32 (82.1%)	67,914	0–24,955,939
M2	85	11 (12.9%)	74 (87.1%)	21,113	0–2,433,288
M3	27	16 (59.3%)	11 (40.7%)	138,248	418–712,112
M4	43	6 (14.0%)	37 (86.0%)	24,239	0–586,663
M5	17	2 (11.8%)	15 (88.2%)	22,084	0–2,562,258
M6	6	0 (0%)	6 (100%)	18,921	228–66,796
M7	9	0 (0%)	9 (100%)	15,158	0–341,398
Total	237	45 (19.0%)	192 (81.0%)	31,537	0–24,955,939

sWT1 expression was preferentially observed in the M3 FAB type followed by the M0 type and its distribution according to the FAB classification was statistically significant ($p < 0.001$). The highest fWT1 expression was observed in the M3 type. The Bonferroni test revealed significant differences between the M2 and M3 FAB types ($p < 0.01$), and between the M3 and M4 FAB types ($p < 0.05$).

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tating the sWT1 transcript might have caused the lower transcript level in our leukemia cases.

The association of sWT1 and fWT1 expressions according to the FAB classification is shown in Table 2. sWT1 expression was preferentially observed in the M3 FAB type (16/27, 59.3%) followed by the M0 type (3/11, 29.3%) and its distribution according to the FAB classification was statistically significant ($p < 0.001$ by the Pearson's χ^2 test). Furthermore, the median WBC count in sWT1-expressing cases (6.9×10^9/L; range, 0.6–372) was significantly lower than in non-expressing cases (19.1×10^9/L; range, 0.7–315) ($p = 0.0083$ by the Mann–Whitney *U*-test). This lower WBC count might have been caused by the high prevalence of sWT1 expression in the M3 type, because the median WBC count in the M3 (5.3×10^9/L; range, 0.6–180) was significantly lower than in the other FAB types (19.1×10^9/L; range, 0.8–372) ($p = 0.0011$ by the Mann–Whitney *U*-test). It has been reported that *FLT3* mutation is frequently identified and associated with leukocytosis in AML, particularly in the M3 FAB type. However, the prevalence of *FLT3* mutation was not significantly different between sWT1-expressing (6/16, 37.5%) and non-expressing (3/11, 27.3%) M3 cases. In addition, sWT1 expression was not associated with *NPM1*, *KIT*, *NRAS*, *CEBPA*, *WT1* and *TP53* mutations (Table 1).

We finally examined the prognostic impact of sWT1 expression in AML. In 176 AML patients, except the M3 FAB type, who were treated with the regimens according to the JALSG (Japan adult leukemia study group) protocols, the positivity of sWT1 expression and its expression level did not affect the complete remission (CR) rate (21/24, 84.0% and 114/151, 75.5% in sWT1 expressing- and non-expressing-patients, respectively), overall survival (OS) and disease-free survival (DFS). Furthermore, sWT1 expression did not affect the CR rate, OS and DFS in 27 M3 FAB types who were treated with the all-*trans* retinoic acid-combined chemotherapy. We also examined the prognostic impact of fWT1 expression in AML, while the high expression of fWT1 did not affect the CR rate, OS and DFS both in AML except the M3 FAB type and in the M3 FAB type.

Although it has been suggested that sWT1 might have more oncogenic properties than fWT1 [6], the sWT1 expression was not implicated in the prognosis and did not stratify AML into a distinct entity in our study. Further analysis is required to clarify whether a lower level of sWT1 expression was actually involved in the pathophisiology of leukemia cells.

Conflict of interest

All authors declare no competing financial interests or conflict of interest.

Acknowledgments

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Contributions. T.N., H.K. and Y.I. designed the research; Y.I. performed the laboratory work for this study; Y.I. and H.K. analyzed the data; H.K. and T.N. wrote the paper and all authors reviewed the manuscript.

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ORIGINAL ARTICLE

FLT3/ITD regulates leukaemia cell adhesion through α4β1 integrin and Pyk2 signalling

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Abstract

Internal tandem duplication of FMS-like receptor tyrosine kinase 3 (FLT3/ITD) within its juxtamembrane domain is a frequent mutation in adult acute myeloid leukaemia (AML). This mutation causes constitutive activation of FLT3 and is associated with poor prognosis. The high relapse rate of FLT3/ITD-positive AML might be partly because of insufficient eradication of slow-cycling leukaemic stem cells in the bone marrow microenvironment. β1 integrin mediates haematopoietic stem and progenitor cell homing along with their retention in the bone marrow and also inhibits haematopoietic proliferation and differentiation. Here, we demonstrate that inhibition of FLT3/ITD kinase activity by a FLT3 selective inhibitor named FI-700 decreases affinity of α4β1 integrin to soluble VCAM-1. α4β1 integrin deactivation by FI-700 is independent of Rap1, which is the critical regulator of integrin inside-out signalling. In addition, selective inhibition of FLT3/ITD induces Pyk2 dephosphorylation together with the inhibition of phosphatidylinositol-3-kinase (PI3K)/Akt pathway. Both wild-type and ITD-FLT3 proteins co-immunoprecipitated with β1 integrin and Pyk2 indicating the signal crosstalk between FLT3, β1 integrin and Pyk2. These results collectively indicated that the inhibition of FLT3 kinase might contribute not only to the induction of apoptosis, but also to the leukaemia cell detachment from the bone marrow microenvironment in the treatment of AML.

Key words leukaemia; FLT3; α4β1 integrin; Pyk2

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FLT3 (FMS-like receptor tyrosine kinase 3) is a member of the class III receptor tyrosine kinase (RTK) and is preferentially expressed in haematopoietic stem cells as well as in the brain, placenta and liver (1, 2). The FLT3 ligand (FL) is expressed either as a membrane-bound or as a soluble form by bone marrow stromal cells. It stimulates the stem cells on its own or in cooperation with other cytokines (3). The FL-FLT3 signal transduction plays an important role in survival, proliferation and differentiation of haematopoietic stem cells. Internal tandem duplication of FLT3 (FLT3/ITD) within its juxtamembrane domain occurs in 15–35% of adults in AML (4). This mutation causes constitutive activation of FLT3 and is associated with poor prognosis (4). The high relapse rate is in part because of the insufficient eradication of slow-cycling leukaemic stem cells in the bone marrow niche.

Adhesion molecules such as $\alpha4\beta1$ integrin (VLA4) and CD44 are crucial for the persistence of leukaemic cells in the bone marrow microenvironment (5, 6). $\beta1$ integrins mediate haematopoietic stem and progenitor cell homing along with their retention in the bone marrow and also inhibit haematopoietic proliferation and differentiation. Having no intrinsic kinase activity, integrins recruit intracellular kinases, such as the focal adhesion kinase (FAK) or the related proline-rich tyrosine kinase 2 (Pyk2), to initiate signal transduction (7). Here, we demonstrate that inhibition of FLT3/ITD kinase activity decreases the affinity of $\alpha4\beta1$ integrin to VCAM-1. On the other hand, selective inhibition of FLT3/ITD causes Pyk2 dephosphorylation together with inhibition of phosphatidylinositol-3-kinase (PI3K)/Akt pathway. Both wild-type (wt) and ITD-FLT3 co-immunoprecipitated with $\beta1$ integrin and Pyk2. These results suggest that there is signal crosstalk between FLT3/ITD and $\beta1$ integrin, and the possibility that FLT3 inhibition can act as an anti-adhesion therapy against leukaemic cells.

Materials and methods

Cells and reagents

NAMO-2 cells were established from a 20-yr-old woman with acute myelomonocytic leukaemia and maintained as described previously (8). Mouse stromal NamoF cells were established from a KSN nude mouse as described previously (8). NAMO-2 was cocultured with NamoF as described previously (8). MOLM-13, a cell line of acute monocytic leukaemia with FLT3/ITD, was made available by Dr Yoshinobu Matsuo (Fujisaki Cell Center, Okayama, Japan) (9). pBJ1-$\alpha4$ and $\beta1$ integrin vectors were generously provided by professor Yoshikazu Takada (University of California Davis School of Medicine, CA, USA). Recombinant human FLT3 ligand and soluble VCAM-1/Fc (sVCAM-1/Fc) chimera protein were purchased from R & D systems (Minneapolis, MN, USA). Recombinant murine interleukin 3 was a generous gift from Kirin Brewery (Tokyo, Japan). FI-700, a selective inhibitor of FLT3 (10) was synthesized by Kyowa Hakko Kirin Co. Ltd. (Tokyo, Japan). The other reagents were purchased from Sigma (St Louis, MO, USA) unless otherwise noted.

Patients and samples

The diagnosis of AML was based on the French-American-British classification. Bone marrow samples from the patients with AML were subjected to Ficoll-Hypaque (Pharmacia LKB, Uppsala, Sweden) density-gradient centrifugation. We confirmed that all samples contained more than 90% leukaemia cells and cryopreserved them in liquid nitrogen before analysis. Informed consent was obtained from all the patients to use their samples for banking and molecular analysis, and approval for these studies was obtained from the Ethics Committee of Nagoya University, Nagoya, Japan.

Screening of the ITD mutation of the *FLT3* gene

High-molecular-weight DNA was extracted from the samples using the standard method. FLT3/ITD was examined by amplification of the JM domain from exon 14 to 15, followed by electrophoresis on an agarose gel as previously reported (11).

Detection of high-affinity $\alpha4\beta1$ integrin by flow cytometry

$\alpha4\beta1$ integrin activation was quantified as previously described (12). In brief, treated cells were suspended in 100 μL of assay buffer (Hanks balanced salt solution; 1 mM $MgCl_2$), at a concentration of 1×10^6 cells/mL, and were incubated at 37°C with 20 μg/mL sVCAM-1/Fc or non-immune human IgG for 30 min at 37°C. Cells were fixed by adding 10 μL of CellFix (BD Biosciences, Sparks, MD, USA) at 25°C for 20 min. Bound sVCAM-1/Fc was detected by adding 2 μL of goat anti-human IgG, Fcγ fragment-phycoerythrin (PE) for 30 min at 4°C. As a positive control for high-affinity $\alpha4$ integrin, 1 mM $MnCl_2$ was added to the complete media. As a negative control, 5 mM of EDTA was added to the media. The relative fluorescence intensity was measured by FACSCalibur (BD Biosciences), and the data are normalized and presented as per cent of maximum. The % of Max is the number of cells in each bin divided by the number of cells in the bin that contains the largest number of cells according to FlowJo algorithms (Tree Star, Inc. Ashland, OR, USA) (13).

Immunoprecipitation

pBJ1-$\alpha4$ integrin, pBJ1-$\beta1$ integrin and pcDNA-FLAG-Pyk2 were cotransfected with either pMKIT-wtFLT3 or pMKIT-FLT3/ITD to COS-7 cell by Effectene reagent (Qiagen, Santa Cruz, CA, USA). Cells were lysed in immunoprecipitation buffer containing 10 mM Tris (pH 7.4), 140 mM NaCl, 1% Triton X-100, 10 μg/mL aprotinin and leupeptin, and 1 mM of PMSF. Lysates were precleared with 30 μL of protein G-Sepharose (GE Healthcare UK Ltd, Buckinghamshire, UK), for 1 hr at 4°C on a rotating platform. Two μg of anti-FLAG M2 monoclonal antibody (Sigma) or control mouse IgG was added to the supernatant and incubated overnight at 4°C. Twenty-five microlitres of protein G-Sepharose

beads were added to the immune complexes and gently rotated for 1 hr at 4°C. The beads were then washed twice with a wash buffer containing 10 mM Tris [(pH 7.4), 140 mM NaCl, 0.1% Triton X-100] followed by a third wash with 10 mM Tris [(pH 7.4), 140 mM NaCl] and fourth wash with 50 mM Tris (pH 6.8). The bound immunocomplexes were recovered by boiling in Laemmli sample buffer for 5 min. The samples were then run on 8% SDS–PAGE and transferred to polyvinylidene difluoride (PVDF) membranes (Bio-Rad Laboratories, Hercules, CA, USA). Proteins were detected with the following antibodies: rabbit anti-FLT3 polyclonal antibody (Santa Cruz Biotechnology; Santa Cruz, CA, USA); rabbit anti-β1 integrin polyclonal antibody (Millipore Corporation, Billerica, MA, USA); and anti-FLAG M2 monoclonal antibody followed by HRP-coupled goat anti-rabbit or mouse Ig (Jackson ImmunoResearch Laboratories Inc., West Grove, PA, USA) using ECL-plus (Amersham Biosciences, Piscataway, NJ, USA).

Akt and Pyk2 phosphorylation assay

Treated cells were lysed with lysis buffer [50 mM NaCl, 50 mM Tris–HCl (pH 7.4), 0.5% Nonidet P-40, 2 mM $MgCl_2$, 10% glycerol, 100 μM PMSF, 10 μg/mL aprotinin, 10 μg/mL leupeptin with Sigma phosphatase inhibitor 2 (Sigma)]. The samples were then run on SDS–PAGE and transferred to PVDF membranes. Proteins were detected with total Akt and Akt phospho-Ser473 antibodies (Cell Signaling Technology, Inc., Danvers, MA, USA), total Pyk2 (BD Biosciences), phospho-Tyr402 of Pyk2 (Cell Signaling) followed by HRP-coupled goat anti-rabbit or mouse Ig (Jackson) using ECL-plus (Amersham Biosciences).

Rap1 activation assay

Rap1 activation was measured as described elsewhere (14). The treated cells were washed with ice-cold PBS briefly and then lysed with ice-cold lysis buffer [200 mM NaCl, 50 mM Tris–HCl (pH 7.4), 1% Nonidet P-40, 2 mM $MgCl_2$, 10% glycerol, 1 mM PMSF, 10 μg/mL aprotinin, 10 μg/mL leupeptin, 1 mM vanadate, 1 mM DTT] was added, and cell lysates were vortexed vigorously. Lysates were kept on ice for 1 min and centrifuged for 5 min at 16, 000 **g** at 4°C. GST-RalGDS-RBD (Ral guanine nucleotide dissociation stimulator Ras-binding domain) (2 μg/μL) was precoupled to glutathione beads. Approximately 40 μL of beads was incubated with 300 μL of cell lysate and incubated for 30 min on a rotating wheel at 4°C. After coupling, GST-RalGDS-RBD-coated beads were washed three times with 1 mL of lysis buffer, and 20 μL of sample buffer was added and boiled (5 min). As a loading control for total Rap1, 20 μL of the cell lysate was supplemented with 10 μL of sample buffer. The samples were run on 13% SDS–PAGE and transferred to PVDF. Rap1 was detected by a polyclonal rabbit Ab (Santa Cruz Biotechnology) and

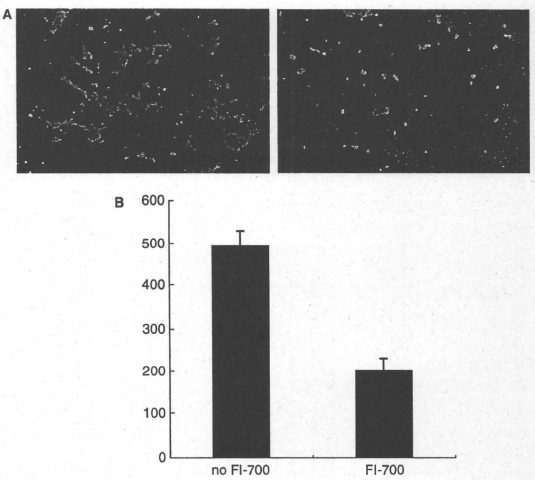

Figure 1 FI-700 induces detachment of NAMO-2 cells from stromal cells. (A) NAMO-2 cells were cocultured with NamoF cells. FLT3 selective inhibitor FI-700 was added at a final concentration of 500 nM, and adhesion was inhibited within 1 hr. The NAMO-2 cells adhering to NamoF cells are observed. The representative figures from six independent experiments are shown. (B) The number of adherent NAMO-2 cells per field was calculated. Bars denote the mean ± SEM of adherent NAMO-2 cells of six independent experiments. $*P < 0.02$.

HRP-coupled goat anti-rabbit Ig (Jackson) using ECL-plus (Amersham Biosciences).

Statistical analysis

Results are reported as the mean ± standard error of the mean (SEM). Levels of significance were determined by the two-sided Student's *t*-test. $P < 0.05$ was accepted as statistically significant.

Results

FLT3/ITD regulates adhesiveness of NAMO-2 cells on stroma cells

NAMO-2, a cell line of acute myelomonocytic leukaemia with FLT3/ITD, was cultured on NamoF cells. Cells were then treated with 500 nM of FLT3 selective inhibitor FI-700 (10). The concentration of FI-700 was decided as described previously (15). The addition of FI-700 inhibited adhesion of NAMO-2 cells on NamoF cells within 1 hr (Fig. 1A & B). FI-700 did not induce apoptosis of NAMO-2 cells under this condition (data not shown) suggesting that FI-700 downregulates certain adhesion molecules.

Inhibition of FLT3/ITD reduces the α4β1 integrin affinity to sVCAM-1 in cell lines

Next, we investigated the role of FLT3/ITD signalling in the regulation of basal α4β1 activity. A flow cytometry assay was used to quantify the kinetics of sVCAM-1/Fc binding to NAMO-2 (Fig. 2A) and MOLM-13 (Fig. 2B), both bearing FLT3/ITD. Preincubation of both cells with FLT3 selective inhibitor FI-700 (500 nM, 1 hr)

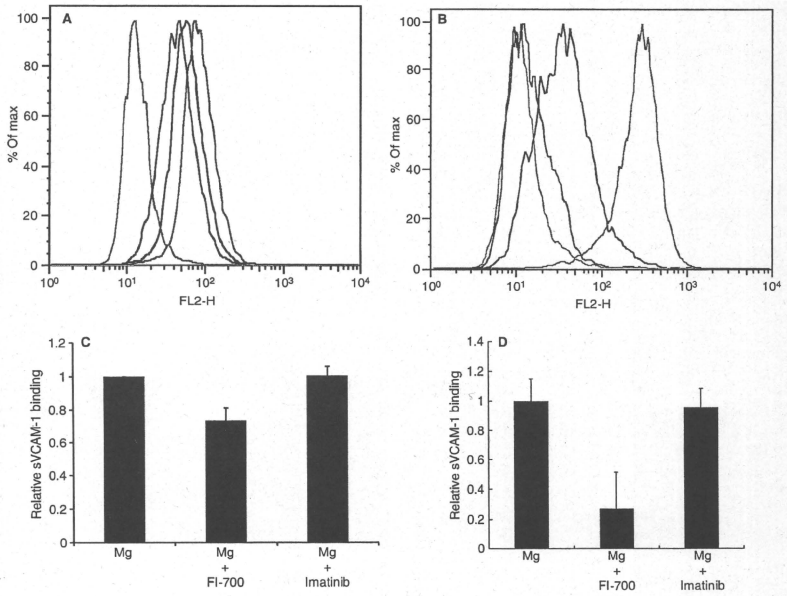

Figure 2 FI-700 reduces sVCAM-1/Fc binding of α4 integrin in the cell lines with FLT3/ITD. NAMO-2 (A) or MOLM-13 (B) cells were pretreated with or without FI-700 (500 nM) for 1 hr, and bound sVCAM-1/Fc was quantified. Cells incubated with 1 mM $MnCl_2$ (orange) and 5 mM EDTA (green) for 60 min at 37°C served as positive and negative controls, respectively. Cells incubated with 1 mM $MgCl_2$ are shown in red and those incubated with FI-700 (A, B) are shown in blue line. The *y*-axis represents maximal cell numbers (%). Representative flow cytometry profiles of three independent experiments are shown. (C, D) Relative sVCAM-1 binding of NAMO-2 (C) and MOLM-13 cells (D) with $MgCl_2$ and inhibitors is shown. Bars denote the mean ± SEM of cells of three independent experiments. $*P < 0.02$.