reported that the presence of an HLA allele mismatch, especially in some specific combinations, significantly affects the outcome of serologically HLA-matched unrelated HSCT.[35] Therefore, the indications for HSCT from an unrelated donor with an HLA allele mismatch should be considered with great caution, especially in standard-risk and older patients.

Recently, minimal residual disease assays are increasingly involved in the evaluation of treatment response for ALL,[36] and the prevalence of minimal residual disease after the induction therapy or early consolidation therapy has been demonstrated as an important prognostic factor. In the current study, we considered only hematological response, and minimal residual disease status was not included in risk stratification. Minimal residual disease status should be taken into account in the future analysis.

In this study, the median duration from achieving CR1 to unrelated HSCT without relapse was 270 days, which precluded HSCT in CR1 in 30% of patients after a decision to perform HSCT (mainly due to early relapse). This duration was 4 months longer than the duration from achieving CR1 to related HSCT without relapse in our previous study, as the coordination process for an unrelated donor through JMDP requires a longer duration. A meta-regression analysis by Yanada *et al.*[11] showed that the proportion of patients who actually underwent allogeneic HSCT among patients with a donor was positively correlated with survival. The coordination process for a JMDP donor is currently getting shorter, and, as a consequence, the efficacy of unrelated HSCT in CR1 may increase.

The low incidence of severe GVHD has been demonstrated in Japanese patients,[37,38] and this might have influenced the superior outcome of unrelated HSCT in CR1 in our analysis. Therefore, caution should be paid when the current results are applied to patients of other origins.

In conclusion, to improve the probability of long-term survival, myeloablative HSCT from a genetically HLA-A, -B, -DRB1 allele-matched unrelated donor in CR1 is recommended for patients, aged 21–54 years, who lack an HLA-matched sibling donor. Even when we considered QOL, the superiority of unrelated HSCT was confirmed in the overall population and in all of the subgroups. However, recent improvements in treatment strategies, like high-intensified chemotherapy, may change this result.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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ORIGINAL ARTICLE

Effect of related donor availability on outcome of AML in the context of related and unrelated hematopoietic cell transplantation

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Although allogeneic hematopoietic cell transplantation (HCT) from a related donor is effective therapy for younger patients with AML, it remains unknown how the availability of a related donor affects the outcome when unrelated HCT is a treatment option for patients without a related donor. To address this issue, we retrospectively analyzed 605 cytogenetically non-favorable AML patients younger than 50 years for whom a related donor search was performed during first CR (CR1). The 4-year OS was 62% in 253 patients with a related donor and 59% in 352 patients without a related donor ($P = 0.534$). Allogeneic HCT was performed during CR1 in 62% and 41% of patients with and without a related donor, respectively. Among patients transplanted in CR1, the cumulative incidence of non-relapse mortality was significantly higher in patients without a related donor ($P = 0.022$), but there was no difference in post-transplant OS between the groups ($P = 0.262$). These findings show the usefulness of unrelated HCT in younger patients with cytogenetically non-favorable AML who do not have a related donor. The extensive use of unrelated HCT for such patients may minimize the potential disadvantage of lacking a related donor.

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Keywords: AML; allogeneic hematopoietic cell transplantation; donor; related transplantation; unrelated transplantation; first CR

INTRODUCTION

Owing to the strong anti-leukemic effect of pre-transplant conditioning therapy in combination with the post-transplant GVL effect, allogeneic hematopoietic cell transplantation (HCT) is currently the most powerful method for preventing relapse of AML.[1] However, the efficacy of allogeneic HCT is compromised by a high risk of treatment-related mortality, which raises the question of whether allogeneic HCT is truly beneficial for AML patients who are in their first CR (CR1). Historically, this question has been investigated in prospective studies that used biologic assignment according to donor availability, in which patients with an HLA-identical sibling donor were assigned to allogeneic HCT, whereas those without an HLA-identical sibling donor were assigned to chemotherapy and/or autologous HCT.[2–8] If we combine the results from those studies, we find that allogeneic HCT during CR1 confers a survival advantage in patients with cytogenetically intermediate and unfavorable risk.[8–10] However, such 'donor vs no-donor' studies do not provide an accurate picture of clinical practice, because an HLA-identical sibling is not the only donor source and a substantial proportion of patients without a related donor receive allogeneic HCT from an unrelated donor.

To examine how related donor availability affects the outcome of AML in a situation where unrelated HCT is a treatment option for patients without a related donor, we retrospectively analyzed cytogenetically non-favorable AML patients under the age of 50 years for whom a related donor search was conducted during CR1. The main objectives of this study were to assess the difference in survival according to related donor availability in terms of (1) overall outcome, (2) outcome after allogeneic HCT in CR1 (that is, comparison between related and unrelated HCT) and (3) outcome after first relapse following chemotherapy. We also looked at how unrelated HCT was incorporated into the treatment strategy in our patient cohort.

PATIENTS AND METHODS

Patients

Adults with AML who had achieved CR1 were retrospectively registered in a Japanese nationwide AML database, which formed the basis of this study. Seventy institutions contributed patients to the database. Patients were eligible if they were younger than 50 years, were diagnosed with AML from 1999 to 2006 according to the World Health Organization (WHO) classification,[11] had achieved CR with one or two courses of chemotherapy, and had a related donor search performed during CR1. We excluded patients with acute promyelocytic leukemia and core-binding factor AML, as well as those whose pre-treatment cytogenetic results were not available. Patients who underwent haploidentical HCT were also excluded. Overall, 605 patients fulfilled these criteria, and thus were subjected to

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subsequent analyses. Information was collected and compiled with regard to patient-related factors (that is, age and sex), disease-related factors (that is, cytogenetics, WBC count and dysplasia in morphology), number of induction courses, related donor availability, and clinical outcome. For patients who underwent allogeneic HCT, complementary information on HCT (that is, interval from CR1 to HCT, disease status at time of HCT, conditioning regimen and donor source) was also collected. Patients were considered to have a related donor if HLA typing identified a matched or one Ag-mismatched family donor. Unrelated donor selection was based on matching at the level of resolution available at the time of transplantation. This study was approved by the Institutional Review Board at the National Cancer Center Hospital.

Statistical analysis

Distributions of patient characteristics between groups were compared by using the χ^2 test for categorical variables and the Wilcoxon rank-sum test for continuous variables. The probabilities of OS and relapse-free survival (RFS) were estimated by the Kaplan–Meier method, with differences between groups qualified by the log-rank test. Relapse and non-relapse mortality (NRM) were considered as competing risk events for each other. The probabilities of relapse and NRM were estimated by the cumulative incidence functions, and differences between groups were qualified by the Gray test. The Cox proportional hazards regression model was used for univariate and multivariate analyses, and a hazard ratio (HR) was calculated in conjunction with a 95% confidence interval (CI). All statistical analyses were performed with SPSS software version 11.0.1 (SPSS, Chicago, IL, USA) and R software version 2.13.0 (The R Foundation for Statistical Computing).

RESULTS

Patient characteristics

Of the 605 patients eligible for analysis, a related donor was found for 253 patients (42%) during CR1. There were no significant differences between the groups in the distribution of baseline characteristics, with the exception of WBC count (Table 1). Figure 1 shows the patient flow with respect to related donor availability, allogeneic HCT in CR1 and relapse. Among the 253 patients with a related donor, 157 (62%) underwent allogeneic HCT in CR1 (156 from a related donor and 1 from an unrelated BM donor). Of the 352 patients without a related donor, allogeneic HCT was performed during CR1 in 146 patients (41%), of whom 109 and 37 received unrelated BMT and umbilical cord blood (UCB) transplantation, respectively. In all, 96 patients with a related donor and 206 patients without a related donor did not receive allogeneic HCT during CR1. Among them, 25 (26%) and 49 (24%) patients experienced early relapse within 6 months after achievement of CR1. Autologous HCT was performed during CR1 in 5 and 14 patients with and without a related donor, respectively.

Characteristics of patients who underwent allogeneic HCT in CR1

The characteristics of patients who underwent allogeneic HCT in CR1 are summarized according to related donor availability in Table 2. The two groups were well balanced in terms of baseline characteristics. However, patients without a related donor were more likely to receive two courses of induction therapy instead of one course ($P = 0.023$). The interval from CR1 to transplantation differed significantly between the groups (Figure 2), with a median interval of 3.7 months for patients with a related donor vs 5.9 months for patients without a related donor ($P < 0.001$). Myeloablative conditioning was used in 89% and 88% of patients with and without a related donor, respectively.

Outcome after CR1 according to related donor availability

The median follow-up of surviving patients was 4.4 years (range, 0.1–9.7), and the 4-year OS was 60% for the entire population. Figure 3 shows Kaplan–Meier survival estimates for patients with and without a related donor. The 4-year OS was 62% in patients with a related donor and 59% in patients without a related donor, with no significant difference detected ($P = 0.534$). Similar results were obtained when the analysis was restricted to patients with unfavorable cytogenetic risk (51% vs 44% at 4 years, $P = 0.213$) or those with intermediate cytogenetic risk (67% vs 67% at 4 years, $P = 0.744$). In the multivariate analysis, cytogenetics and number of induction courses were identified as factors that were significantly associated with OS, whereas related donor availability had no significant impact (Table 3).

Table 1. Patient characteristics

	Related donor + N = *253*	*Related donor −* N = *352*	P-*value*
Age, years			0.547
Median	34	35	
Range	16–49	16–49	
Sex			0.232
Male	127 (50%)	194 (55%)	
Female	126 (50%)	158 (45%)	
Cytogenetic risk by SWOG			0.74
Intermediate	153 (60%)	211 (60%)	
Unfavorable	72 (28%)	94 (27%)	
Unknown	28 (11%)	47 (13%)	
WBC count, × 10⁹/L			0.049
Median	13.8	18	
Range	0.6–794.0	0.5–410.7	
Dysplasia			0.9
No	204 (81%)	285 (81%)	
Yes	49 (19%)	67 (19%)	
No. of induction courses			0.186
1 Course	196 (77%)	256 (73%)	
2 Courses	57 (23%)	96 (27%)	
Allogeneic HCT			<0.001
CR1	157 (62%)	146 (41%)	
CR2	27 (11%)	44 (13%)	
Other disease phases	38 (15%)	63 (18%)	
Not performed	31 (12%)	99 (28%)	

Abbreviations: CR1 = first CR; CR2 = second CR; SWOG = Southwest Oncology Group.

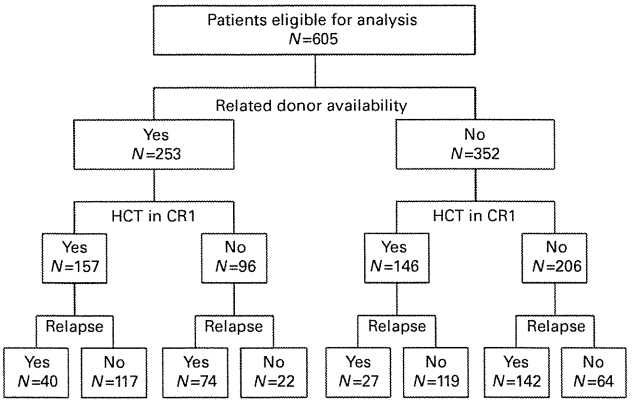

Figure 1. Flow diagram of patients.

Table 2. Characteristics of patients who underwent allogeneic HCT in CR1

	Related donor+ N = *157*	*Related donor−* N = *146*	P-*value*
Age, years			0.441
Median	34	35	
Range	16–49	16–49	
Sex			0.252
Male	80 (51%)	84 (58%)	
Female	77 (49%)	62 (42%)	
Cytogenetic risk by SWOG			0.178
Intermediate	97 (62%)	78 (53%)	
Unfavorable	40 (25%)	47 (32%)	
Unknown	20 (13%)	21 (14%)	
WBC count, × 10^9/L			0.644
Median	12.7	11.5	
Range	0.9–794.0	0.6–410.7	
Dysplasia			0.729
No	118 (75%)	112 (77%)	
Yes	39 (25%)	34 (23%)	
No. of induction courses			0.023
1 Course	115 (73%)	89 (61%)	
2 Courses	42 (27%)	57 (39%)	
Interval from CR1 to HCT, days			<0.001
Median	113	178	
Range	0–620	14–770	
Type of donor			<0.001
Related	156 (99%)	0 (0%)	
Unrelated, BM	1 (1%)	109 (75%)	
Unrelated, cord blood	0 (0%)	37 (25%)	
Type of conditioning			0.969
Myeloablative	140 (89%)	128 (88%)	
Reduced-intensity	14 (9%)	13 (9%)	
Not specified	3 (2%)	5 (3%)	

Abbreviations: CR1 = first CR; HCT = hematopoietic cell transplantation; SWOG = Southwest Oncology Group.

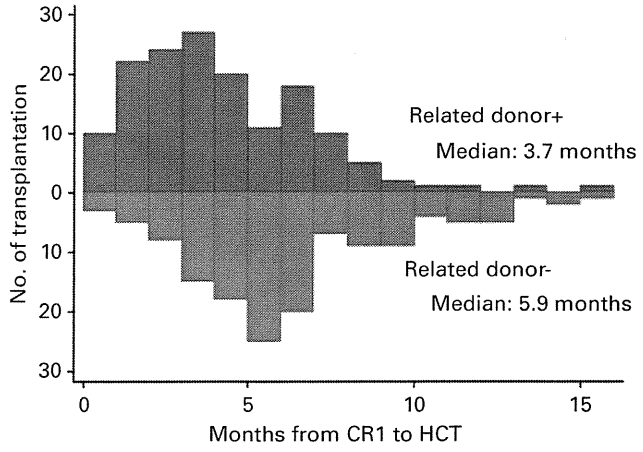

Figure 2. Interval from CR1 to HCT in patients who underwent allogeneic HCT during CR1. Patients with (Related donor +, $N = 157$) and without a related donor (Related donor −, $N = 146$) are shown separately. A full color version of this figure is available at the *Bone Marrow Transplantation* journal online.

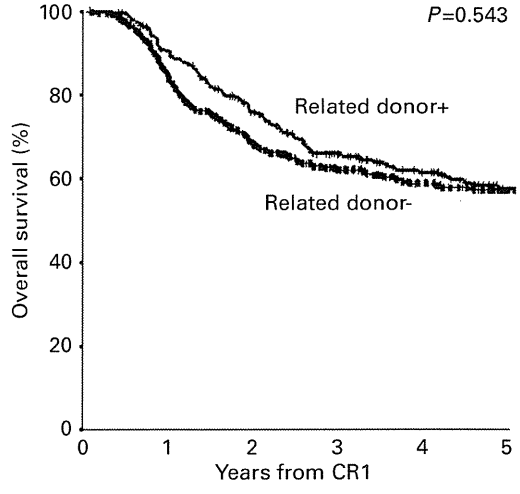

Figure 3. Kaplan–Meier curves for OS after CR1 according to related donor availability. All patients with (Related donor +, $N = 253$) and without a related donor (Related donor −, $N = 352$) are compared.

Outcome after allogeneic HCT in CR1 according to related donor availability

Figure 4 compares post-transplant OS between patients with and without a related donor who underwent allogeneic HCT during CR1. There was no difference in OS between the groups: the 4-year OS was 65% in patients with a related donor and 61% in patients without a related donor ($P = 0.262$). The cumulative incidence of NRM in patients with a related donor was significantly lower than that in patients without a related donor (13% vs 21% at 4 years, $P = 0.022$). In terms of relapse, patients with a related donor appeared to show a higher incidence, but the difference was not statistically significant (26% vs 21% at 4 years, $P = 0.292$). OS with unrelated BMT was superior to that with UCB transplantation (66% vs 48% at 4 years, $P = 0.044$): the former was equivalent to the result with related HCT ($P = 0.897$), whereas the latter was worse ($P = 0.003$). Related HCT from a matched ($N = 140$) and one Ag-mismatched donor ($N = 16$) showed no difference in OS (66% vs 56% at 4 years, $P = 0.304$).

Effect of allogeneic HCT during CR1 in patients with or without a related donor

To examine how allogeneic HCT in CR1 impacted RFS and OS, we performed separate multivariate analysis for patients with and without a related donor. In this analysis, HCT was considered as a time-dependent covariate, and adjustments were made for all of the variables listed in Table 3 except for related donor availability. In patients with a related donor, allogeneic HCT in CR1 was associated with superior RFS (HR, 0.28; 95% CI, 0.19–0.41) and OS (HR, 0.65; 95% CI, 0.43–0.98). In patients without a related donor, allogeneic HCT in CR1 had favorable effect on RFS (HR, 0.58; 95% CI, 0.41–0.82) and OS (HR, 0.82; 95% CI, 0.56–1.19), although the effect on OS did not reach statistical significance.

Outcome after first relapse following chemotherapy according to related donor availability

Among patients who did not undergo allogeneic HCT in CR1, 74 patients with a related donor and 142 patients without a related

Table 3. Factors associated with OS

	Univariate analysis			*Multivariate analysis*		
	HR	*(95% CI)*	P-*value*	*HR*	*(95% CI)*	P-*value*
Related donor availability						
Yes	1		—	1		—
No	1.09	(0.84–1.40)	0.534	1.08	(0.83–1.39)	0.576
Age						
As a numerical variable (per 1 year)	1	(0.99–1.01)	0.799	1	(0.99–1.01)	0.926
Sex						
Male	1		—	1		—
Female	0.98	(0.76–1.27)	0.899	1	(0.78–1.30)	0.979
Cytogenetic risk by SWOG						
Intermediate	1		—	1		—
Unfavorable	1.92	(1.45–2.54)	<0.001	2.00	(1.51–2.65)	<0.001
Unknown	1.72	(1.18–2.50)	0.005	1.52	(1.04–2.22)	0.031
WBC count						
As a numerical variable (per 10×10^9/L)	1.02	(1.00–1.03)	0.035	1.01	(1.00–1.03)	0.065
Dysplasia						
No	1		—	1		—
Yes	0.7	(0.49–1.00)	0.052	0.71	(0.50–1.02)	0.061
No. of induction courses						
1 Course	1		—	1		—
2 Courses	2.44	(1.88–3.17)	<0.001	2.46	(1.88–3.21)	<0.001

Abbreviations: CI = confidence interval; HR = hazard ratio; SWOG = Southwest Oncology Group.

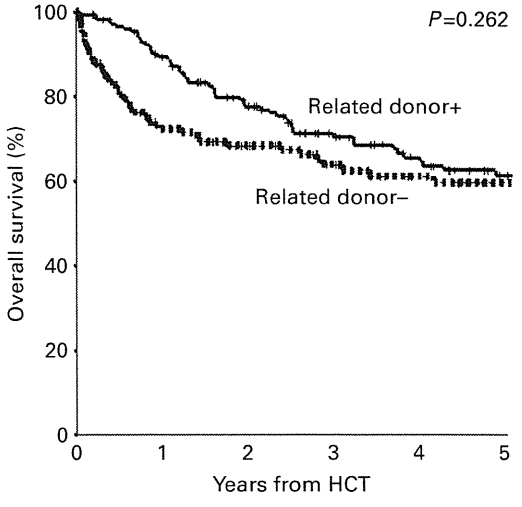

Figure 4. Kaplan–Meier curves for OS after allogeneic HCT in CR1 according to related donor availability. Patients with (Related donor+, $N = 157$) and without a related donor (Related donor−, $N = 146$) who underwent allogeneic HCT in CR1 are compared.

donor experienced relapse (Figure 1). After relapse, 65 (88%) patients with a related donor received allogeneic HCT (62 from a related donor, 2 from an unrelated BM donor and 1 from UCB), as did 107 (75%) patients without a related donor (63 from an unrelated BM donor, 42 from UCB and 1 from a related donor who had not been included in the initial related donor search; information was missing for 1 patient). In all, 27 patients with a related donor and 44 patients without a related donor received allogeneic HCT during CR2. For patients who experienced relapse without having received allogeneic HCT in CR1, the 4-year OS after relapse was 33% in patients with a related donor and 33% in patients without a related donor ($P = 0.245$).

DISCUSSION

The outcome of unrelated HCT has recently improved primarily due to the introduction of high-resolution HLA-typing technology and improvements in supportive care. In addition, the growth of unrelated donor registries as well as the increased use of UCB grafts has increased the chance of finding an unrelated donor.[1] These advances have made unrelated HCT a more feasible option for patients who lack a related donor. As our analyses were based on a nationwide multicenter survey, the finding that 41% of patients without a related donor received unrelated HCT during CR1 reflects the widespread use of unrelated HCT in Japan. On the other hand, in patients with a related donor, the proportion of patients who underwent allogeneic HCT during CR1 reached 62%. This value was comparable to or only slightly lower than the HCT compliance rates reported in previous donor vs no-donor studies, where allogeneic HCT was offered to all patients with a related donor as per the study protocol.[2–8] These findings show that allogeneic HCT, from both related and unrelated donors, was actively incorporated into the treatment strategy in our patient population.

When we take into account that patients with core-binding factor AML were excluded from our study, the 60% 4-year OS for the entire cohort appears quite favorable. Recently, the Japan Adult Leukemia Study Group reported results from a prospective study (designated AML201) for newly diagnosed AML patients, in which standard-dose and high-dose cytarabine (AraC)-based regimens were compared for post-remission therapy.[12] In that study, for patients younger than 50 years, the 5-year OS was 66%

with standard-dose AraC consolidation and 62% with high-dose AraC consolidation. As the AML201 study included patients with core-binding factor AML (28% of the total population), and the patients in that study were selected according to the pre-defined inclusion and exclusion criteria, it is remarkable that a comparable survival rate was achieved in our patients. The active use of allogeneic HCT not only in patients with a related donor but also in those without a related donor likely contributed to the favorable overall outcome of our patients.

In contrast to the results of meta-analysis studies of the prospective donor vs no-donor comparison,[8–10] our patients with and without a related donor had comparable OS. Similar results were obtained if the outcome was compared in terms of RFS (data not shown). The most likely explanations for this result is that up to 41% of our patients without a related donor proceeded to unrelated HCT during CR1, and that OS after allogeneic HCT in CR1 did not differ between patients with and without a related donor. NRM is a major obstacle to the success of unrelated HCT. Early studies showed less satisfactory results with unrelated HCT because of a high incidence of NRM.[13,14] However, according to more recent data, comparable outcomes have been reported for related and unrelated HCT in AML patients.[15–18] In our study, the cumulative incidence of NRM in patients undergoing unrelated HCT was significantly higher than that in those undergoing related HCT (21% vs 13%, $P=0.022$), but the NRM rate of 21% with unrelated HCT appears to be within the acceptable range. The benefits of unrelated HCT may be increased by reducing NRM with the aid of stricter matching between donor and patient, increasing the use of reduced-intensity conditioning, and applying better supportive care. Recently, several groups have conducted prospective donor vs no-donor studies for AML patients with high-risk features by expanding the type of donor to include unrelated donors.[19–21] Notably, despite a limited number of patients in each study, they showed significantly superior OS in patients with a donor, as well as comparable OS in patients undergoing related and unrelated HCT.[19–21] These prospective studies also support the usefulness of unrelated HCT in younger AML patients with non-favorable cytogenetics. Although our multivariate analysis showed that the degree to which allogeneic HCT had favorably affected outcome was less marked in patients without a related donor compared with those with a related donor, unrelated HCT could be considered a reasonable treatment option if a related donor is not available.

When our data are interpreted, it should be remembered that this study was an observational study, not an interventional study. The decision of whether or not to proceed to allogeneic HCT could be confounded by multiple factors, and early relapse, for example, did not seem to be a main cause for not having undergone allogeneic HCT during CR1 in our study. Adjusting for known confounding factors by using a multivariate analysis cannot guarantee that biases are removed. Thus, the results presented here need to be interpreted cautiously. Although we acknowledge such a limitation, our data showed that related donor availability did not significantly affect OS in younger patients with cytogenetically non-favorable AML. We consider this was because 41% of the patients without a related donor underwent unrelated HCT during CR1, and the outcome after transplantation was comparable between related and unrelated HCT. These results show the usefulness of unrelated HCT in this patient population when they do not have a related donor. The extensive use of unrelated HCT for such patients may minimize the potential disadvantage of lacking a related donor.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

ACKNOWLEDGEMENTS

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Author contributions: MY designed the study, interpreted data, and wrote the manuscript; SK prepared the data file, performed the analysis and interpreted data; TY was primarily responsible for the study design, data analysis and interpretation of the data; NU, SM, HK, KU, TK, MW, KN, SY, Y Nawa, JT, HT, Y Nakamura, SF and FK obtained the patients' data and interpreted data; NE designed the study and interpreted data; IM reviewed the cytogenetic reports and interpreted data; and TF interpreted data and helped to write the manuscript.

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bjh research paper

Outcome after first relapse in adult patients with Philadelphia chromosome-negative acute lymphoblastic leukaemia

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Summary

To analyse the outcome of adult patients who developed a first relapse of acute lymphoblastic leukaemia (ALL), we collected the clinical data of 332 patients with Philadelphia-chromosome (Ph) negative ALL, aged 16–65 years, who relapsed after first complete remission (CR1) between 1998 and 2008 in 69 institutions all over Japan, including 58 patients who relapsed after allogeneic haematopoietic stem cell transplantation (Allo-HSCT) in CR1. The overall survival (OS) was 43·4% at 1 year, and 16·3% at 5 years from relapse in patients who received chemotherapy alone in CR1. Among patients who relapsed after chemotherapy alone in CR1, 123 (52·5%) achieved a second remission (CR2) following salvage chemotherapy, of whom 62 subsequently underwent Allo-HSCT during CR2. Allo-HSCT in CR2 was significantly associated with better OS. Moreover, the type of salvage chemotherapy influenced OS from relapse. A doxorubicin, vincristine, and predonisone-based (AdVP-type) regimen was related to better OS in patients with longer CR1 (more than 1 year), but was related to worse OS in patients with shorter CR1. In conclusion, the prognosis of patients with relapsed Ph-negative ALL is poor. Allo-HSCT after a first relapse could improve the prognosis. Selection of the optimal salvage chemotherapy might depend on the duration of CR1.

Keywords: acute lymphoblastic leukaemia, first relapse, second remission, salvage chemotherapy, allogeneic haematopoietic stem cell transplantation.

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The complete remission (CR) rate of adult patients with acute lymphoblastic leukaemia (ALL) has improved to about 90% with modern intensive chemotherapy. However, many patients eventually relapse, and the long-term leukaemia-free survival rate is only 30–40% (Litzow, 2009). Many relapsed patients receive various salvage therapies after the first relapse, and several studies have reported that 38–56% of relapsed patients can achieve second remission (CR2; Giona *et al*, 1997; Thomas *et al*, 1999; Camera *et al*, 2004; Tavernier *et al*, 2007; Cornelissen *et al*, 2009). While allogeneic haematopoietic stem cell transplantation (Allo-HSCT) in CR2 is considered to be the only curative strategy, early relapse and/or organ dysfunction after salvage chemotherapy and the lack of a suitable donor often prevent Allo-HSCT at this stage. Therefore, the prognosis of adult patients with relapsed ALL is extremely poor (Fielding *et al*, 2007; Tavernier *et al*, 2007; Oriol *et al*, 2010; Gokbuget *et al*, 2012). We collected clinical data after the first relapse in adult patients with Philadelphia chromosome (Ph)-negative ALL who were treated in institutions all over Japan, and performed a retrospective analysis to clarify the prognosis and prognostic factors for the outcome in relapsed patients. Patients with Ph-positive ALL were not included in our analysis because the outcome of treatment in these patients has improved dramatically since tyrosine kinase inhibitors became available (Ottmann & Pfeifer, 2009).

Methods

Data source

We retrospectively collected clinical data of patients with Ph-negative ALL, aged 16–65 years, who relapsed after achieving first remission (CR1) between 1998 and 2008. Patients with lymphoblastic lymphoma were not included. Patients had received either chemotherapy alone, autologous stem cell transplantation (Auto-HSCT), or Allo-HSCT in CR1. Patients who did not achieve remission with chemotherapy but eventually achieved remission after HSCT were excluded. CR was defined based on haematological findings, and not on molecular findings. The data collected included clinical information about the disease at both diagnosis and relapse, the content of therapies both at diagnosis and after relapse, and the clinical course. This study was approved by the Institutional Review Board of Saitama Medical Centre, Jichi Medical University.

Statistical considerations

Differences between groups were examined using the Fisher's exact test for categorical variables and the Mann-Whitney U-test for continuous variables. Overall survival (OS) was calculated using the Kaplan-Meier method. To evaluate the influence of factors for OS, the log-rank test and proportional-hazards modelling were used for univariate and multivariate analyses, respectively. In a univariate analysis, the impact of Allo-HSCT in CR2 was assessed by a landmark analysis that was limited to patients who achieved CR2 and survived without relapse for at least 68 d, which was the median duration between the achievement of CR2 and the performance of Allo-HSCT in CR2. A post-hoc multicomparison test using the Holm method was performed for comparisons among three groups.

For the multivariate analysis, we included all covariates at first, and used the backward stepwise selection of covariates. Finally, P values of less than 0·05 were considered statistically significant. Potential confounding factors at diagnosis that were considered in the analysis were sex, phenotype of ALL, white blood cell (WBC) count, karyotype (patients with t (4;11) or complex karyotype (5 or more chromosomal abnormalities) were considered high-risk, and were compared to those with other karyotypes), presence of central nervous system (CNS) invasion of ALL, intensity of chemotherapy, and duration between the diagnosis and the achievement of CR1. Moderate-intensity regimens at diagnosis were defined as those that included both high-dose cytarabine and high-dose methotrexate, and the other regimens were regarded as conventional-intensity. In addition, potential confounding factors at relapse that were considered in the analysis were relapse year, age, duration of CR1, sites of relapse, WBC count, and additional chromosomal abnormalities. Allo-HSCT in CR2 was also included as a time-dependent covariate in the multivariate analysis.

We specifically evaluated the influence of salvage chemotherapy after relapse on OS. Salvage chemotherapy regimens were categorized into three types. The first was combination chemotherapy based on conventional doses of doxorubicin, vincristine, and predonisone (AdVP-type). The second type was fractionated cyclophosphamide,

vincristine, doxorubicin, and dexamethasone alternating with high doses of methotrexate and cytarabine (hyperC-VAD/MA; Kantarjian *et al*, 2000), and the third type included high-dose cytarabine (Kantarjian *et al*, 1986), the combination of mitoxantrone, etoposide, and cytarabine (MEC; Amadori *et al*, 1991), and the combination of high-dose cytarabine and mitoxantrone (HAM; Lejeune *et al*, 1990). Potential confounding and selection biases for these three kinds of salvage regimens were considered through the Fisher's exact test.

All statistical analyses were performed with EZR (Saitama Medical Centre, Jichi Medical University; Kanda, 2012),

Table I. Patient characteristics.

	All patients (n = 332)	Relapse after CTx alone (n = 270)	Relapse after Allo-HSCT (n = 58)	P value*
Patient characteristics at diagnosis				
Age, years; median (range)	34 (15–65)	37 (15–65)	28 (16–58)	0·006
Sex				
Male	165 (50%)	132 (49%)	30 (52%)	0·773
Female	167 (50%)	138 (51%)	28 (48%	
Phenotype				
T	65 (20%)	47 (17%)	17 (29%)	0·038
B	253 (76%)	213 (79%)	37 (64%)	
Other	6 (2%)	4 (1%)	2 (3%)	
WBC, $\times 10^9$/l; median (range)	11·1 (0·6–759·7)	11·0 (0·6–759·7)	14·4 (0·6–537·9)	0·264
Karyotype				
normal	162 (49%)	138 (51%)	20 (34%)	0·025
t(4;11)	10 (3%)	10 (4%)	0 (0%)	
complex	50 (15%)	36 (13%)	14 (24%)	
others	84 (25%)	66 (25%)	18 (31%)	
CNS invasion				
−	312 (94%)	255 (94%)	53 (91%)	0·238
+	12 (4%)	8 (3%)	4 (7%)	
CTx intensity†				
Conventional	184 (55%)	149 (55%)	32 (55%)	1·0
Moderate	142 (43%)	117 (43%)	25 (43%	
Days from diagnosis to CR1; median (range)	38 (14–337)	37 (14–337)	42 (21–143)	0·081
Patient characteristics at relapse				
Relapse year				
Upto end 2003	152 (46%)	127 (47%)	21 (36%)	0·147
2004 onwards	180 (54%)	143 (53%)	37 (64%)	
Follow-up after relapse, days; median (range)	1344 (12–3689)	1381 (12–3689)	1001 (726–2799)	0·540
Duration of CR1				
Median (range) (d)	290 (15–7162)	246 (15–7162)	465 (33–2185)	<0·001
<1 year	193 (58%)	171 (63%)	22 (38%)	
≥ 1 year	135 (41%)	96 (36%)	35 (60%)	
Sites of relapse				
BM included	288 (87%)	237 (88%)	47 (81%)	<0·001
CNS alone	29 (9%)	28 (10%)	1 (2%)	
others	13 (4%)	4 (1%)	9 (16%)	
Age, years; median (range)	35 (16–65)	37 (16–65)	29 (18–59)	0·011
WBC, $\times$ 10^9/l; median (range)	4·5 (0·6–331·6)	4·4 (0·6–331·6)	5·1 (1·2–137·3)	0·168
Additional chromosomal abnormality				
−	231 (70%)	198 (73%)	29 (50%)	0·01
+	52 (16%)	37 (14%)	15 (26%)	
Patients that achieved CR2, n	163 (53·6%)	136 (54·0%)	26 (54·2%)	0·753

*P value was calculated based on the comparison of patients who relapsed after CTx alone in CR1 to those who relapsed after Allo-HSCT in CR1. Differences between these two groups were examined using the Fisher's exact test for categorical variables and the Mann–Whitney U-test for continuous variables.

†Moderate-intensity regimens included both high-dose cytarabine and high-dose methotrexate.

CTx, chemotherapy; Allo-HSCT, allogeneic haematopoietic stem cell transplantation; WBC, white blood cell; CNS, central nervous system; CR, complete remission; BM, bone marrow.

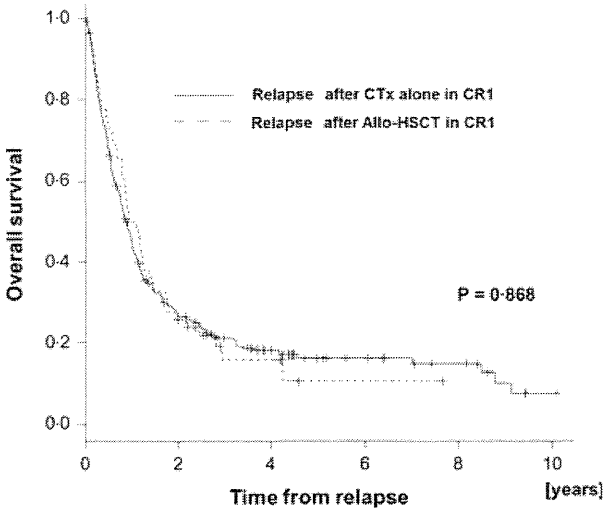

Fig 1. Overall survival from relapse. Overall survival (OS) from relapse in relapsed patients after chemotherapy (CTx) alone in first complete remission (CR1) and in those after allogeneic haematopoietic stem cell transplantation (Allo-HSCT) in CR1. The OS rate was not significantly different between these two groups.

which is a graphical user interface for R (The R Foundation for Statistical Computing, version 2.13.0).

Results

Patient characteristics

A total of 332 patients, treated in 69 institutions all over Japan, were included in this study. The characteristics of the patients are summarized in Table I. Their median age at relapse was 35 years, and 165 patients were male. The median duration of CR1 was 290 d (range 15–7162 d), and the median follow-up time after relapse was 1344 d (range 12–3689 d). Two-hundred and seventy patients relapsed after chemotherapy alone in CR1, and 58 and 4 patients relapsed after Allo- and Auto-HSCT in CR1, respectively. When the patients who relapsed after chemotherapy alone in CR1 were compared to those who relapsed after Allo-HSCT in CR1, the distribution of the phenotype and karyotypes of leukaemic cells were significantly different. In addition, older age at relapse, shorter duration of CR1, and lower rate of the appearance of additional chromosomal abnormalities at relapse were observed in patients who relapsed after chemotherapy alone in CR1.

Overall survival (OS) from relapse in the 270 patients who received chemotherapy alone in CR1 was 43·4% at 1 year and 16·3% at 5 years, while OS from relapse in the 58 patients who received Allo-HSCT in CR1 was 50·0% at 1 year and 10·6% at 5 years. The OS rate was not significantly different between these two groups (hazard ratio (HR): 0·97, 95% confidence interval (CI): 0·64–1·29, $P = 0·868$; Fig 1).

Outcome of relapsed patients after chemotherapy alone in CR1

Among patients who relapsed after chemotherapy alone in CR1, 43% had received moderate-intensity regimens at diagnosis, such as hyperCVAD/MA (Kantarjian *et al*, 2000) and regimens used in the Japan Adult Leukaemia Study Group (JALSG) ALL201 studies (C000000063 for older patients and C000000064 for younger patients; Sakura *et al*, 2012). The remaining patients had received conventional-intensity regimens, which were mainly used in JALSG ALL93 (Takeuchi *et al*, 2002) and ALL97 (Jinnai *et al*, 2010) studies.

Among the 270 patients who relapsed after chemotherapy alone in CR1, 234 received salvage chemotherapy, and 123 of these 234 (52·5%) achieved a second complete remission (CR2; Fig 2). Sixty-two of these 123 patients underwent Allo-HSCT in CR2, 16 patients from a human leucocyte antigen (HLA)-matched related donor, 6 from an HLA-mismatched related donor, and 30 from an unrelated donor. The remaining 10 received unrelated cord blood. Fifty-five of them received myeloablative conditioning. The median duration between the achievement of CR2 with salvage chemotherapy and Allo-HSCT in CR2 was 68 d (range 10–276 d). OS from CR2 was significantly better in patients who under-

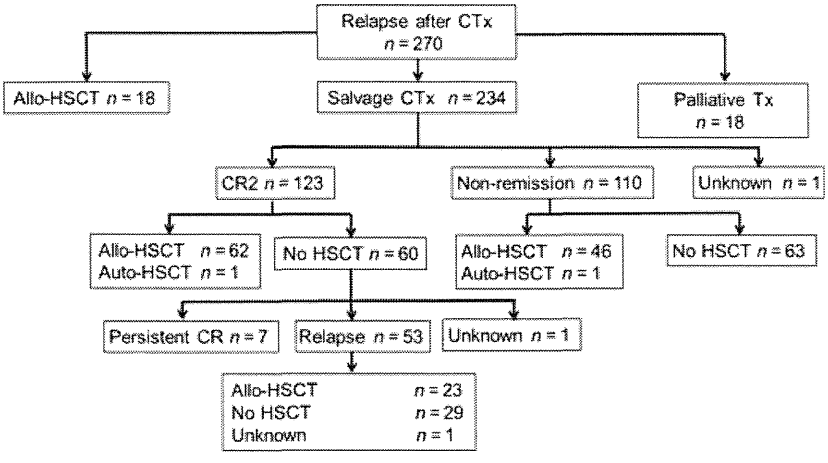

Fig 2. Clinical course of relapsed patients after chemotherapy alone in the first complete remission. Among the 270 patients who relapsed after chemotherapy (CTx) alone in first complete remission (CR1), 234 received salvage CTx, and 123 of these 234 patients (52·5%) achieved a second CR (CR2). Eighteen patients underwent allogeneic haematopoietic stem cell transplantation (Allo-HSCT) directly after relapse without salvage CTx. Tx, treatment; Auto-HSCT, autologous haematopoietic stem cell transplantation

went Allo-HSCT in CR2 than in those who did not (73·7% vs. 50·0% at 1 year and 43·9% vs. 11·0% at 5 years, respectively; HR: 0·43, 95% CI: 0·26–0·72, $P = 0·001$) by a landmark analysis that was limited to patients who were alive without disease at 68 d after they had achieved CR2 (Fig 3). The results of a Mantel-Byar analysis that evaluated the influence of Allo-HSCT in CR2 were similar to that of a landmark analysis using the log-rank test (HR: 0·49, 95% CI: 0·31–0·77, $P = 0·002$). In a multivariate analysis using the backward stepwise selection of covariates, younger age at relapse (younger than 36 years), lower WBC count at relapse (less than 10×10^9/l), and Allo-HSCT in CR2 treated as a time-dependent covariate were associated with better OS among patients who achieved CR2 following salvage chemotherapy (Table II).

We further analysed the effect of each salvage regimen that patients received after the first relapse. Patients who received one of the three types of salvage regimen below were included in this analysis. Those who had isolated CNS relapse were excluded. Seventy-five patients received the AdVP-type salvage regimen, and 58 received the hyperCVAD/MA regimen (Kantarjian *et al*, 2000). The other 46 patients received a high-dose cytarabine (Kantarjian *et al*, 1986; $n = 22$), MEC (Amadori *et al*, 1991; $n = 14$), or HAM (Lejeune *et al*, 1990; $n = 10$) regimen. The type of salvage regimen did not significantly influence OS from relapse (Fig 4A). In the univariate analyses, a longer duration of CR1 (more than 1 year; $P = 0·003$), the intensity of chemotherapy at diagnosis ($P < 0·001$), and the relapse year (before 31 December 2003 or after 1 January 2004; $P = 0·008$) were significantly associated with the selected salvage regimen. Therefore, the patients included in this analysis were stratified into two groups according to the duration of CR1, intensity of chemotherapy at diagnosis, and relapse year, respectively. The duration of CR1 was less than 1 year in 96 patients and longer than 1 year in 71 patients. In patients who had a shorter duration of CR1, the type of salvage regimen influenced OS ($P = 0·002$; Fig 4B). In the post-hoc test, patients who received the AdVP-type regimen had worse OS, compared to those who received the hyperCVAD/MA regimen ($P = 0·040$) or other regimens ($P < 0·001$). The type of salvage regimen also influenced OS in patients who had a longer duration of CR1 ($P = 0·048$; Fig 4C). In contrast, the post-hoc test indicated that patients who received the AdVP-type regimen were associated with better OS with borderline significance, compared to those who received the hyperCVAD/MA regimen ($P = 0·066$). When patients were stratified into two groups according to the intensity of chemotherapy at diagnosis or the relapse year, the type of salvage regimen did not influence OS ($P = 0·733$ and 0·843, in patients who received conventional- and moderate-intensity chemotherapy at diagnosis, respectively, and $P = 0·131$ and 0·892, in patients who relapsed before 31 December 2003 and after 1 January 2004, respectively).

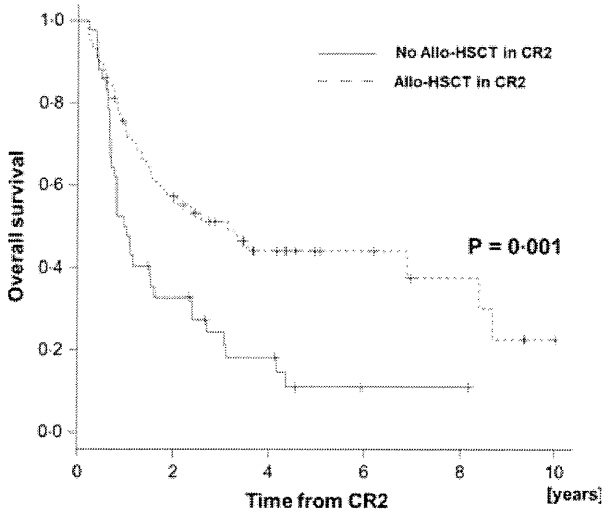

Fig 3. Overall survival from the second complete remission (CR) in patients who relapsed after chemotherapy alone in the first CR. Among the 123 patients who achieved a second complete remission (CR2) following salvage chemotherapy, 62 underwent allogeneic haematopoietic stem cell transplantation (Allo-HSCT) in CR2. The median duration between the achievement of CR2 and Allo-HSCT in CR2 was 68 d. The overall survival (OS) rate from CR2 was significantly better in patients who underwent Allo-HSCT in CR2, according to a landmark analysis that was limited to patients who were alive without disease at 68 d from the achievement of CR2.

Table II. Prognostic factors in patients who relapsed after chemotherapy alone in first complete remission (CR1) and achieved second CR (CR2) following salvage chemotherapy.

	Multivariate analysis	
	Hazard ratio (95% CI)	*P* value
Allo-HSCT in CR2*		
+	0·49 (0·31–0·79)	0·003
−		
Age at relapse		
>35 years	1·83 (1·18–2·82)	0·007
≤35 years		
WBC count at relapse		
≥10 × 10^9/l	2·87 (1·67–4·95)	<0·001
<10 × 10^9/l		

*Allo-HSCT in CR2 was treated as a time-dependent covariate.
CI, confidence interval; allo-HSCT, allogeneic haematopoietic stem cell transplantation; CR, complete remission; WBC, white blood cell.

Outcome of Allo-HSCT in non-remission after relapse

In relapsed patients who had received chemotherapy alone in CR1, 18 patients directly underwent Allo-HSCT after relapse without salvage chemotherapy and 46 underwent Allo-HSCT in non-remission (NR) following failed salvage chemotherapy. OS from Allo-HSCT in patients who underwent Allo-HSCT in NR after salvage chemotherapy was 39·1% at 1 year and 20·7% at 5 years. OS from Allo-HSCT in patients who underwent Allo-HSCT directly after relapse was 38·9% at 1 year and

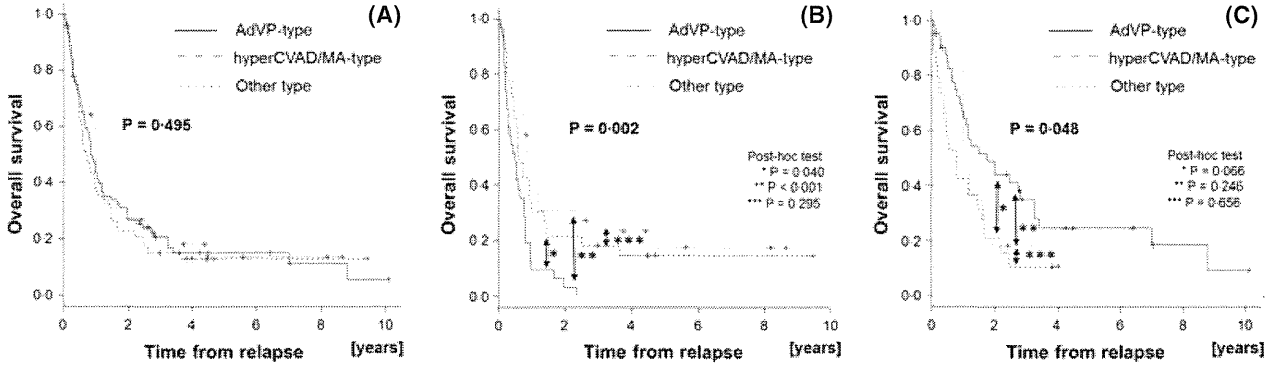

Fig 4. Overall survival from relapse in patients who received chemotherapy alone in the first complete remission according to the salvage chemotherapy regimen. The type of salvage regimen did not significantly influence overall survival (OS) from relapse (A). However, if patients were stratified into two groups according to the duration of the first complete remission (CR1), the type of salvage regimen was significantly associated with OS. In patients who had a shorter duration of CR1 (<1 year), an AdVP-type regimen (doxorubicin, vincristine, and predonisone-based) was associated with a worse OS (B), and in patients who had a longer duration of CR1 (more than 1 year), an AdVP-type salvage regimen tended to result in a better OS (C). hyperCVAD/MA, fractionated cyclophosphamide, vincristine, doxorubicin, and dexamethasone alternating with high doses of methotrexate and cytarabine.

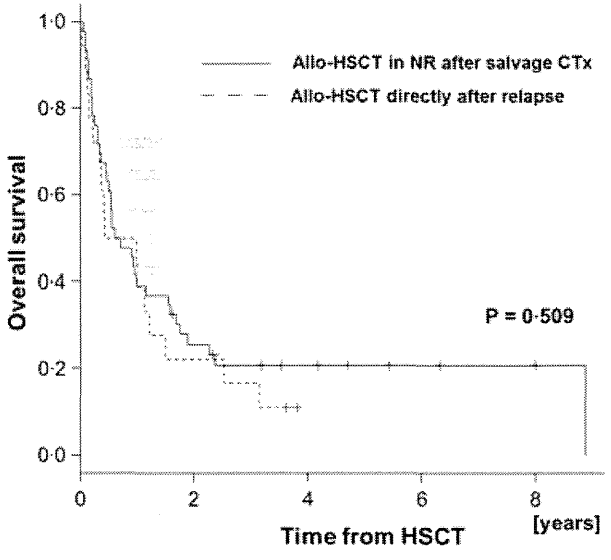

Fig 5. Overall survival from allogeneic haematopoietic stem cell transplantation in non-remission after relapse. In relapsed patients who received chemotherapy alone in the first complete remission, there was no difference in overall survival from allogeneic haematopoietic stem cell transplantation (Allo-HSCT) between patients who underwent Allo-HSCT in non-remission (NR) following salvage chemotherapy (CTx) and patients who underwent Allo-HSCT directly after relapse without salvage CTx.

11·1% at 5 years. There was no difference in OS from Allo-HSCT between these two groups (HR: 0·81, 95% CI: 0·17–1·45, $P = 0·51$; Fig 5).

Discussion

Several large clinical studies have demonstrated the outcomes of a relatively large number of patients with ALL who relapsed after uniform chemotherapy regimens (Fielding *et al*, 2007; Tavernier *et al*, 2007; Oriol *et al*, 2010; Gokbuget *et al*, 2012), and the results of uniform salvage chemotherapy regimens have been reported as clinical trials, each of which included a small number of relapsed patients with ALL (Giona *et al*, 1997; Koller *et al*, 1997; Montillo *et al*, 1997; Weiss *et al*, 2002; Camera *et al*, 2004; Specchia *et al*, 2005). The present study investigated the prognosis of relapsed patients with Ph-negative ALL, based on the clinical data of 332 patients from 69 institutions all over Japan. These patients had received various kinds of treatment strategies before and after relapse according to their respective institution, and therefore, these patients should reflect the more general population of relapsed patients with ALL.

Overall survival (OS) at 5 years from relapse was 16·3% in patients who received chemotherapy alone in CR1 and 10·6% in patients who received Allo-HSCT in CR1, and Allo-HSCT in CR1 did not influence the outcome after relapse in our study. These outcomes were comparable to those in three previous reports of large clinical studies (Fielding *et al*, 2007; Tavernier *et al*, 2007; Oriol *et al*, 2010; Table III). Recently, Gokbuget *et al* (2012) reported the outcome of 547 relapsed patients in the German Multicentre Study Group for Adult ALL (GMALL). The OS at 5 years from relapse in patients who had received chemotherapy alone in CR1 was significantly better than that in patients who had received Allo-HSCT in CR1 (28% vs. 15%, $P < 0·001$). This difference might be attributed to the high rate (75%) of Allo-HSCT after relapse in patients who had received chemotherapy alone in CR1. In our study, Allo-HSCT was performed in 55% of the patients who relapsed after they had received chemotherapy alone in CR1. Allo-HSCT in CR2 was associated with a better prognosis after the achievement of CR2, and in addition, some of the patients who received Allo-HSCT even in NR after salvage chemotherapy showed long-term survival (the OS was 20·7%

Table III. Comparison of several studies regarding the outcome of relapsed patients with acute lymphoblastic leukaemia (ALL).

	Patients (*n*)	Rate of CR2 (%)	5-year OS	Prognostic factors for improved OS
LALA-94 (Tavernier *et al*, 2007)	421	44%	8%	Allo-HSCT after relapse CR1 > 1 year Platelet count at relapse >100 × 10^9/l
MRC UKALL12/ECOG2993 (Fielding *et al*, 2007)	609	N.M.	7%	Age at relapse <20 years CR1 > 2 years
PETHEMA trials (Oriol *et al*, 2010)	263	45%	10%	Age at relapse <30 years CR1 > 2 years
GMALL trials (Gokbuget *et al*, 2012)*	547	42% after CTx alone† 23% after Allo-HSCT‡	28% after CTx alone† 15% after Allo-HSCT‡	No Allo-HSCT in CR1 Extramedullary relapse (other than CNS) CR1 > 18 months§ Age at relapse§ (15–25 years vs. 26–45 years vs. 46–55 years) CR after 1st salvage CTx§ Allo-HSCT at any stage§
This study	332	53% after CTx alone† 54% after Allo-HSCT‡	16% after CTx alone† 11% after Allo-HSCT‡	HSCT in CR2¶ WBC count at relapse <10 × 10^9/l¶ Age at relapse <35 years¶

*Patients with Philadelphia chromosome-positive ALL were not included in this report from GMALL trials.

†The rate is among patients who relapsed after CTx alone in CR1.

‡The rate is among patients who relapsed after Allo-HSCT in CR1.

§These factors were associated with better OS in patients who relapsed after CTx alone in CR1.

¶These factors were associated with better OS in patients who achieved CR2 following relapse after CTx alone in CR1.

CR1, first complete remission; CR2, second complete remission; OS, overall survival; Allo-HSCT, allogeneic haematopoietic stem cell transplantation; CTx, chemotherapy; CNS, central nervous system; WBC, white blood cell count; LALA-94, Leucémies Aiguës Lymphoblastiques de l'Adulte 94 trial; MRC UKALL12/ECOG2993, Medical Research Council, United Kingdom ALL 12/Eastern Cooperative Oncology Group 2993 trial; PETHEMA, Programme for the Study and Treatment of Haematological Malignancies; GMALL, German Multicentre Study Group for Adult ALL, N. M., not mentioned.

at 5 years). These findings suggested that we should consider Allo-HSCT as much as possible for relapsed patients.

Prognostic factors that were associated with a better OS from CR2 were younger age at relapse, lower WBC count at relapse, and Allo-HSCT in CR2 among patients who relapsed after chemotherapy alone in CR1 and achieved CR2 following salvage chemotherapy. None of the factors at diagnosis was associated with OS after relapse. Age and Allo-HSCT were the common factors observed in other studies (Table III). The duration of CR1 was not associated with better OS in the multivariate analysis in our study. In other studies, the duration of CR1 was associated with not only better OS but also a high rate of achieving CR2 (Thomas *et al*, 1999; Tavernier *et al*, 2007; Oriol *et al*, 2010). Given that our analysis was limited to patients who achieved CR2, the duration of CR1 might not be a significant factor for OS. Although the WBC count at relapse was not analysed in the other studies, it should be considered as an important factor.

We should note that there might be selection bias regarding the performance of Allo-HSCT in CR2, because it depended on each institution's decision. By a multivariate analysis using logistic regression for all covariates, relapse year (after 2003) and younger age at relapse (younger than 36 years) were significantly associated with the performance of Allo-HSCT in CR2. However, a multivariate analysis for OS including relapse year, age at relapse, and Allo-HSCT in CR2 (treated as a time-dependent covariate) as covariates demonstrated that Allo-HSCT in CR2, as well as age at relapse, was still significantly associated with better OS. In addition, there was no significant interactions between Allo-HSCT in CR2 and relapse year and between Allo-HSCT in CR2 and age at relapse ($P = 0{\cdot}36$ and $P = 0{\cdot}97$, respectively).

Comparisons of different salvage chemotherapy regimens have been limited (Thomas *et al*, 1999; Tavernier *et al*, 2007; Oriol *et al*, 2010). The selection of salvage regimens often depends on the condition of the relapsed patient (Garcia-Manero & Thomas, 2001). In our analyses, the duration of CR1, intensity of chemotherapy at diagnosis, and relapse year were factors that were associated with the selection of salvage regimens. AdVP-type salvage chemotherapy was related to a better OS in patients who had a longer duration of CR1 and a worse OS in those who had a shorter duration of CR1. If we consider that many patients had received induction chemotherapy including doxorubicin, vincristine, and steroids, the duration of CR1 might reflect the sensitivity of ALL to the AdVP-type regimen. Patients who had a longer duration

of CR1 might have had ALL that was sensitive to an AdVP-type regimen, and those who had a shorter duration of CR1 might have had ALL that was refractory to an AdVP-type regimen. In patients who relapsed late, the toxicity of moderate-intensity regimens, such as those including high-dose cytarabine, used as the first salvage chemotherapy might offset their effectiveness. Unlike in a previous study (Thomas *et al*, 1999), the type of chemotherapy at diagnosis did not influence OS following each salvage regimen.

In conclusion, the prognosis of adult patients with relapsed Ph-negative ALL is poor. However, Allo-HSCT after the first relapse could improve the prognosis, especially if performed in CR2. The efficacy of different types of salvage chemotherapy might depend on the duration of CR1, and this should be considered in the selection of the salvage regimen.

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Authorship

Author contributions: S.K. wrote the paper. All authors contributed to writing the paper and checked the final version. S.K. and Y.K. designed the study and analysed data. All authors participated in data collection.

Conflict of interest disclosures

The authors declare no competing financial interests.

Supporting Information

Additional Supporting Information may be found in the online version of this article:

Appendix S1. Participating Centres

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ORIGINAL ARTICLE: CLINICAL

Clinical evaluation of WT1 mRNA expression levels in peripheral blood and bone marrow in patients with myelodysplastic syndromes

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Abstract

A study to evaluate WT1 mRNA expression levels in peripheral blood (PB) and bone marrow aspirate (BM) was conducted in 172 patients, including 115 with myelodysplastic syndromes (MDS), in Japan. The level of WT1 mRNA expression was evaluated according to the French–American–British (FAB) and World Health Organization (WHO) classifications (2001, 2008) and using the International Prognostic Scoring System and the WHO Prognostic Scoring System scales. WT1 mRNA expression levels in PB and BM were well correlated ($r = 0.85$), and they tended to increase with disease stage progression and in those at higher risk of leukemic transformation. WT1 mRNA expression can be a useful marker for the diagnosis and risk evaluation of MDS.

Keywords: Myelodysplastic syndromes, WT1 mRNA expression, classification system, peripheral blood, bone marrow

Introduction

Myelodysplastic syndrome (MDS), a clonal disorder of pluripotent hematopoietic stem cells, is a blood disease characterized by dysplasia and ineffective hemopoiesis. Approximately 20–30% of cases of MDS undergo transformation to acute myeloid leukemia (AML) [1].

The expression of Wilms' tumor gene (WT1) has been found to be a new prognostic factor and marker for the detection of minimal residual disease (MRD) in acute leukemia, including AML and acute lymphocytic leukemia (ALL) [2]. A recent study has revealed the clinical relevance of measuring WT1 mRNA for monitoring MRD in AML, primarily due to its high rate of expression (93.9%) in the peripheral blood (PB) of incipient untreated patients with AML, secondarily due to its ability to predict relapse after complete remission (CR), and finally because its levels after consolidation therapy

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show a significant correlation between disease-free survival, overall survival and early relapse [3]. WT1 mRNA expression occurs not only in AML but also in the PB and bone marrow (BM) of patients with MDS [4–9].

Tamaki *et al.* [4] examined the level of WT1 mRNA expression in PB and BM from 57 patients with MDS grouped by the French–American–British (FAB) classification, and 12 patients experienced AML-MDS progression. The results revealed that WT1 mRNA expression in both PB and BM progressively increased with disease stage progression, from refractory anemia (RA), refractory anemia with excess of blasts (RAEB), refractory anemia with excess of blasts in transformation (RAEB-t), and to AML, suggesting the possibility that the WT1 mRNA expression level reflects the disease stage progression of MDS. Particularly, the patient group who developed leukemia from RAEB or RAEB-t within 6 months showed significantly higher WT1 mRNA expression in PB compared with the group who did not [4].

In accordance with that study, Cilloni *et al.* [6] measured WT1 mRNA expression levels in PB and BM from 131 patients with MDS, and found that: (1) WT1 mRNA expression in PB and BM was confirmed in 78% and 65% of patients with RA, respectively; (2) WT1 mRNA expression in PB and BM was confirmed in all patients with RAEB and secondary AML; (3) the level of WT1 mRNA expression increased with disease stage progression; and (4) the WT1 mRNA expression level was well correlated with the International Prognostic Scoring System (IPSS) scores established by Greenberg *et al.* [10].

In addition to the IPSS, the World Health Organization (WHO) Classification-Based Prognostic Scoring System (WPSS) has been proposed as a prognostic scoring system for MDS [11]. The WPSS consists of three characteristics: WHO subtype classification, considered to be important as a prognostic factor; IPSS-based karyotype abnormalities; and transfusion dependency.

Both the IPSS and WPSS require a chromosomal test as a primary parameter. However, because there are cases in which chromosomal abnormalities cannot be determined [12–14], it is necessary to establish molecular- and genetic-based methods to diagnose and determine the prognosis of MDS. The relatively rapid quantitation of WT1 mRNA is considered to be a useful test to determine the prognosis of MDS and has potential for clinical application, to become a novel marker to complement the current IPSS and WPSS criteria. We performed a clinical study in patients with MDS to demonstrate the usefulness of measuring the WT1 mRNA expression level in PB and BM in the diagnosis and treatment of MDS.

Patients and methods

This study was conducted in accordance with the Declaration of Helsinki, and preliminary approval was obtained from the Institutional Review Board or equivalent organization of each participating institution. Explanations of the study protocol were provided to all patients, and written informed consent was obtained from them before study enrollment.

Patients

From December 2008 to September 2009, 175 patients with MDS, suspected MDS and AML-MDS examined at 17 Japanese medical institutions were enrolled in the study. The subjects were 20 years of age or older and entered in the study regardless of gender, inpatient/outpatient status, or presence or absence of treatment. The 175 patients comprised 106 men (age range 27–88 years, average 65.5 years) and 69 women (age range 22–85 years, average 64.5 years). PB and BM samples from each patient were collected on the same day and used for WT1 mRNA measurement. Three of the 175 enrolled patients were excluded because BM could not be collected due to a dry tap or because the subtype could not be diagnosed. A total of 172 patients were therefore included in the final analysis set.

Diagnosis

Diagnosis of MDS was carried out using a central review format based on the FAB classification [15], the 2001 WHO classification [16] and the 2008 WHO classification [17]. Central review of the bone marrow smear-stained specimens, blood smear-stained specimens, iron-stained specimens, and clot hematoxylin and eosin-stained specimens was carried out by two individuals, one each in the Department of Hemato-Oncology, Saitama International Medical Center, Saitama Medical University, and the Department of Laboratory Medicine, Kawasaki Medical School.

WT1 mRNA measurement method

mRNA was extracted from PB leukocytes and BM nucleated cells at SRL, Inc., Tokyo, Japan using the RNeasy Mini-Kit (Qiagen, Valencia, CA), and the amount containing WT1 mRNA was measured at the Research Laboratory, Diagnostic Division, Otsuka Pharmaceutical Co., Ltd., Tokushima, Japan using a WT1 mRNA Assay Kit (Otsuka Pharmaceutical Co., Ltd., Tokyo, Japan). cDNA was synthesized from 1 μg of extracted RNA in a reverse-transcription reaction using random hexamer primers. The amounts of WT1 and GAPDH (glyceraldehyde 3-phosphate dehydrogenase) mRNA were quantitated using real-time polymerase chain reaction (PCR) with a COBAS TaqMan48 analyzer (Roche Diagnostics, Pleasanton, CA), and the respective amounts of WT1 and GAPDH RNA in the sample were calculated by simultaneous reaction with standards of known concentrations.

Method for calculating WT1 mRNA expression

mRNA of the universally expressed housekeeping gene GAPDH was used for correction of variations in the efficiencies of RNA extraction and reverse transcription. As shown in the following formula, the level of WT1 mRNA expression was calculated by dividing the measured amount of WT1 mRNA by the measured amount of GAPDH mRNA and multiplying that value by the average number of copies of GAPDH mRNA found in 1 μg of RNA from PB leukocytes of healthy adults (GAPDH mRNA expression). The average GAPDH mRNA expression in PB leukocytes of healthy adults was reported to be 2.7×10^7 copies/μg RNA based on independent tests in healthy adults [3].

WT1 mRNA expression (copies/μg RNA) = (measured WT1 mRNA [copies/mL]/measured GAPDH mRNA [copies/mL]) × 2.7 × 10^7 (copies/μg RNA)

PB cut-off value

The lower limit of the WT1 mRNA measurement range in the WT1 assay kit is 2500 copies/mL, or 50 copies/μg RNA when converted to copies per microgram of RNA. In this study, a value of 50 copies/μg RNA was set as the cut-off value for WT1 mRNA expression, and a value of 50 or more copies/μg RNA was judged as positive according to the instruction manual of the WT1 mRNA assay kit.

Statistical analysis

The mean ± SD for the log-transformed values of WT1 mRNA expression (copies/μg RNA) was calculated, and then converted back to base 10 and used as the geometric mean. All data below the detection limit were shown as 49 copies/μg RNA. For intergroup comparison of WT1 mRNA expression, a Tukey-Kramer honestly significant difference (HSD) test was performed at the level of significance of $p < 0.05$ using log-transformed values of WT1 mRNA expression (copies/μg RNA). For comparison of WT1 mRNA expression between the aplastic anemia (AA) and RA groups, a Wilcoxon rank-sum test and Steel test were performed at the level of significance of $p < 0.05$ using log-transformed values of WT1 mRNA expression (copies/μg RNA). The Pearson correlation coefficient was used for analysis of each correlation.

Results

As a result of the central review conducted on all 172 patients, 115 were classified as patients with MDS in the FAB classification, excluding chronic myelomonocytic leukemia (CMML). Similarly, 98 patients in the 2001 WHO classification and 97 in the 2008 WHO classification were classified as patients with MDS (Figure 1).

Analytical results based on FAB classification

WT1 mRNA expression in PB and BM

The 172 patients eligible for analysis were categorized by disease type, and their WT1 mRNA expression levels in PB and BM are shown in Table I. The mean WT1 mRNA expression level in the 115 patients with MDS (excluding CMML) was 360 copies/μg RNA in PB and 2240 copies/μg RNA in BM, and these values were the second highest after the values obtained in patients with AML-MDS (PB: 12 600 copies/μg RNA; BM: 33 100 copies/μg RNA). On the other hand, the WT1 mRNA expression level was less than 50 copies/μg RNA in PB and 90–630 copies/μg RNA in BM in patients with AA, idiopathic cytopenia of unknown significance (ICUS), idiopathic thrombocytopenic purpura (ITP), paroxysmal nocturnal hemoglobinuria (PNH), pure red-cell aplasia (PRCA) and erythroid hypoplasia, which were all lower compared with the level in MDS.

The relationship between WT1 mRNA expression in PB and BM was evaluated in all patients. The regression line formula $y = 0.7329x + 1.4407$ was obtained, indicating a strong correlation ($r = 0.85$) (Figure 2).

WT1 mRNA expression in PB and BM for each MDS disease stage

When the WT1 mRNA expression levels in PB and BM were compared for each MDS subtype based on the FAB classification [Figure 3(a)], the level in both increased proportionally with each MDS classification as the disease

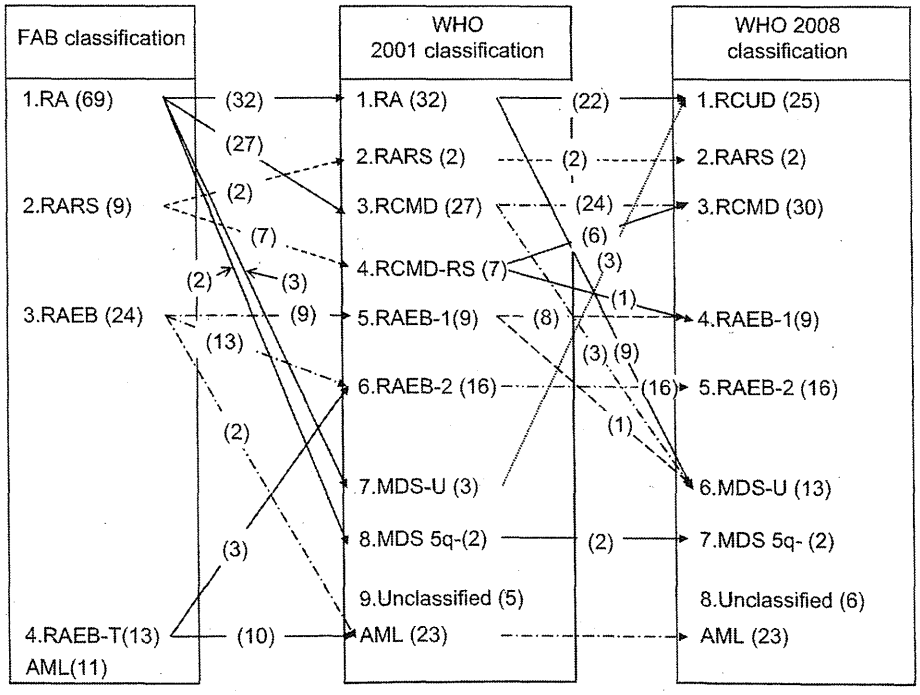

Figure 1. FAB and WHO classification of myelodysplastic syndromes in this study. FAB classification-based MDS subtypes (four subtypes: RA, RARS, RAEB and RAEB-t), 2001 WHO-based MDS subtypes (eight subtypes: RA, RARS, RCMD, RCMD-RS, RAEB-1, RAEB-2, MDS-U and MDS 5q−), 2008 WHO-based MDS subtypes (seven subtypes: RCUD, RARS, RCMD, RAEB-1, RAEB-2, MDS-U and MDS 5q−). Numbers in parentheses represent numbers of patients.