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Day 0 bone marrow pathology of allogeneic hematopoietic stem cell transplantation is a novel prognostic factor in myeloid malignancies

Takahiko Sato[1], Naomi Kawashima[1], Masafumi Ito[2], Yoshiko Atsuta[3], Yusuke Kagaya[1], Aika Seto[1], Takanobu Morishita[1], Nobuaki Fukushima[1], Yukiyasu Ozawa[1], Koichi Miyamura[1]

[1]Department of Hematology, Japanese Red Cross Nagoya First Hospital, Nagoya, Japan, [2]Department of Pathology, Japanese Red Cross Nagoya First Hospital, Nagoya, Japan, [3]Japanese Data Center for Hematopoietic Cell Transplantation, Nagoya, Japan

Abstract

Residual disease (RD) is one of the risk factors for relapse after hematopoietic stem cell transplantation (HSCT) in hematological malignancies. Although recent advances in the technology for detecting minimal/measurable RD, such as multiparameter flow cytometry and quantitative PCR, enable risk stratifications of disease relapse, these examinations still have limitations in routine clinical practice. In this study, we assessed RD in bone marrow (BM) specimens on day 0 of allogeneic HSCT by immunostaining of case-specific leukemic blast markers and analyzed the relationship between day 0 BM status and HSCT outcomes. We analyzed 82 adult HSCT recipients with myeloid malignancies. BM histology of day 0 revealed almost empty marrow with a small number of residual BM cells. However, residual blasts could be detected by immunostaining even for only a few cells. When patients were divided into two groups according to the existence of RD on day 0, those with positive RD showed significantly lower overall survival rate (27% vs. 73%, $P<0.001$) and higher cumulative incidence of relapse (46% vs. 9%, $P=0.006$) at one year compared to those with negative RD. Furthermore, even if they were not in remission at the point of the pre-conditioning evaluation, the patients who achieved negative RD on day 0 showed comparable prognosis with those who maintained remission before conditioning. This study shows the efficacy of day 0 BM pathology of allogeneic HSCT as a prognostic factor that can contribute to clinical decisions on post-transplant strategies.

Key words: bone marrow pathology, allogeneic hematopoietic stem cell transplantation, minimal residual disease, myeloid malignancies, relapse

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Correspondence: Naomi Kawashima, Department of Hematology, Japanese Red Cross Nagoya First Hospital, 3-35, Michishita-cho, Nakamura-ku, Nagoya, Japan, E-mail: nkawashima@med.nagoya-u.ac.jp

Introduction

Allogeneic hematopoietic stem cell transplantation (allo-HSCT) is a curable therapy for hematological malignancies. However, relapse remains an obstacle for patients' survival[1,2]. Minimal/measurable residual disease is considered as one of the risk factors for relapse after allo-HSCT[3,4]. It is widely known that patients without hematological remission have poor outcomes after allo-HSCT[5,6]. In a previous report of refractory acute myeloid leukemia (AML) and myelodysplastic syndrome (MDS), low leukemia burden, less than 5% blasts in bone marrow (BM) with the absence of peripheral blood blasts at the time of transplant, showed better overall survival (OS) than higher leukemia burden with more BM blast counts and circulating blasts[7]. Recent advances in the technology for detecting minimal residual disease including multiparameter flow cytometry, quantitative PCR and next-generation sequencing also enabled identification of poor responders to chemotherapy and provided risk stratifications[8-10]. However, they are based on the evaluation before conditioning treatment in the setting of allo-HSCT.

In patients with residual diseases (RD), BM status at

the point of stem cell infusion, namely after conditioning treatment, potentially differs from that of pre-conditioning assessment, depending upon the response to conditioning chemotherapy and irradiation or proliferative potency of the residual blasts. Therefore, the evaluation of disease status on day 0 marrow could predict post-transplant outcomes and help planning upfront post-transplant strategies for residual diseases. In the present study, we histopathologically evaluated the BM on day 0 of allo-HSCT and its impact on outcomes after allo-HSCT.

Patients and Methods

Patients

We retrospectively analyzed a total of 82 consecutive adult patients who received a first allo-HSCT for AML or MDS with excess blasts (MDS-EB) between 2010 and 2014 in Japanese Red Cross Nagoya First Hospital. Patients with extramedullary diseases were excluded from the study. This study was approved by the ethical committee of our institutional review board and all patients provided informed consent in accordance with the Declaration of Helsinki.

Pathological examinations

BM examinations were performed routinely before conditioning treatment and on day 0 of stem cell infusion. BM clot sections before conditioning treatment and the day of stem cell infusion were examined by histopathology and immunostaining. All specimens were fixed in 10% formalin solution and embedded in paraffin. All slides were stained with hematoxylin and eosin as well as with the naphthol AS-D chloroacetate esterase plus Giemsa double staining (ASD-G) method. Immunostaining was conducted using a Leica Bond-III^{TM} according to instruction manuals (Leica Biosystems, Newcastle, UK). CD7, CD34, CD56, CD117 (c-kit), TdT, and p53 antibodies (Leica Biosystems, Newcastle, UK) were used in this study. Antibodies were selected according to the specific blast tumor phenotypes in individual cases.

Definitions

Detection of a single residual blast by immunostaining in a whole BM clot section on day 0 was defined as positive RD (Day 0-RD+), whereas patients without a residual blast were defined as negative RD (Day 0-RD−). In patients with MDS-EB, dysplasia in residual BM cells was not considered but only the existence of residual blasts by immunostaining was defined as RD in this study. Hematological remission was defined as less than 5% of blasts in BM smear samples evaluated by each physician.

Neutrophil engraftment post allo-HSCT was defined as the achievement of an absolute neutrophil count≥500/mm^3 for three consecutive days. Myeloablative conditioning (MAC) was defined as a conditioning regimen with at least 8 Gy of total body irradiation or busulfan administration over 16 mg/kg. All other regimens were categorized as reduced-intensity conditioning (RIC).

Statistical analyses

Overall survival (OS) was measured from the date of stem cell infusion to the date of death from any cause or the last follow-up (censoring). Survival curves were plotted by the Kaplan-Meier method, and univariate analyses were performed with the log-rank test. Competing risk analyses were used to estimate the cumulative incidence of relapse, and univariate analyses were performed with Gray's test. All variables with $P<0.1$ from univariate analyses were included in the multivariate analyses, which were performed with either the Cox proportional hazard regression model or the Fine-Gray competing risks regression model. All statistical tests were two-sided, and statistical significance was defined as $P<0.05$. All statistical analyses were performed with EZR 1.30 (Saitama Medical Center, Jichi Medical University, Saitama, Japan)[11].

Results

Pathology of day 0 bone marrow

BM clot sections of pre-conditioning and day 0 stained with ASD-Giemsa and anti-CD34 antibody are shown in **Figure 1**. BM histology of Day 0-RD− revealed almost empty marrow with a small number of residual mature granulocytes, erythroblasts, lymphocytes and macrophages compared with normoplastic BM before conditioning treatment (**Figure 1A**). Although BM blasts could be identified by ASD-G staining in few cases, residual blasts were able to be detected even for only a few cells by using immunostaining of case-specific markers in BM of Day 0-RD+ group (**Figure 1B**).

Patient characteristics

Within the 82 patients, 71 (86.6%) were included in Day 0-RD− group and 11 (13.4%) were in Day 0-RD+ group, whose characteristics are summarized in **Table 1**. All of the patients were not in hematological complete remission (nCR) before conditioning treatment in Day 0-RD+ group, whereas 27 of 71 patients (38.0%) in Day 0-RD− group were nCR before conditioning but blast cells were not detected on day 0 ($P<0.001$, **Table 1**). Among the other 44 patients in Day 0-RD− group who were in hematological CR before conditioning, molecular RD was also assessed in BM before conditioning of 19 patients by measuring the expression levels of chimeric transcripts of *CBFB-MYH11* (n=3) and *DEK-NUP214*

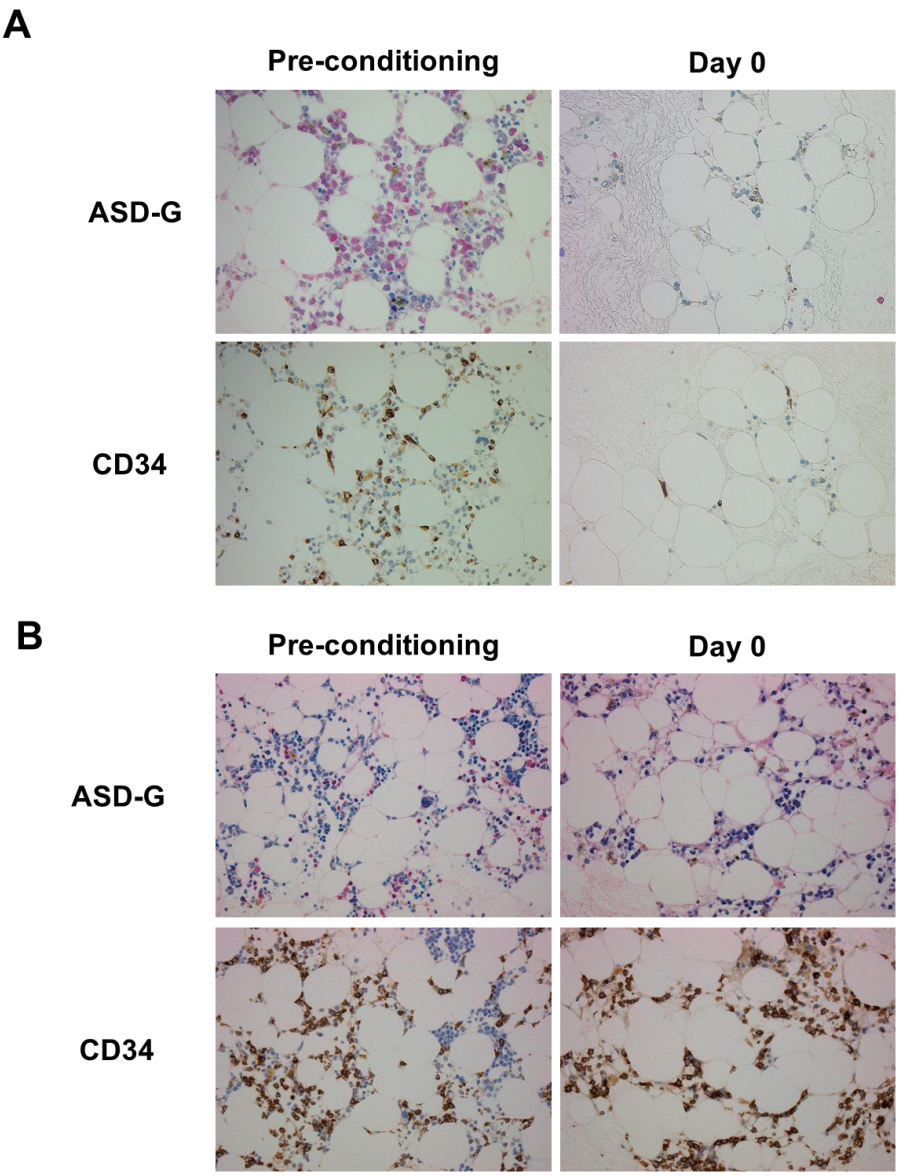

Figure 1. Residual diseases in bone marrow clot sections
BM specimens of pre-conditioning and day 0 of allo-HSCT stained with ASD-Giemsa and CD34 are shown. Representative AML patients with (A) nCR before conditioning and RD− on day 0, and (B) nCR before conditioning and RD+ on day 0. Original magnification×200.
ASD-G, ASD-Giemsa; RD, residual disease.

(n=2), and Wilms tumor 1 (*WT1*) mRNA (n=14). Molecular RD was detectable before conditioning in 13 patients with positive *WT1* mRNA (median 900, range 230-32,000 copies/μg RNA).

The median percentage of BM blasts before conditioning was significantly higher in Day 0-RD+ group than Day 0-RD− group (71% vs. 3%, $P<0.001$), which had no significant correlation with the blast percentage of day 0 marrow (Spearman rank order correlation coefficient, $rs=0.47$). When limited to patients with nCR at pre-conditioning, the median percentage of BM blasts was higher in the Day 0-RD+ group than the Day 0-RD− group in AML (83% vs. 19%, $P=0.01$); however, it was similar between the two groups in MDS-EB (17% vs. 11%, $P=0.28$).

Positive residual disease in Day 0 BM was an independent risk factor for survival and relapse

The intensity of conditioning regimen had no significant impact on day 0 marrow status ($P=0.53$). Eleven of 27 patients (41%) including 6 of 13 MDS patients (46%) in nCR before conditioning received MAC and achieved Day 0-RD− status, whereas 5 of 11 (45%) in nCR before conditioning received MAC and stayed in Day 0-RD+.

Neutrophil engraftment was achieved in 78 patients (95%) at a median of 16 days (range 10-42 days) and no significant difference was observed based upon the day 0 BM status ($P=0.38$).

Median follow-up period of survivors was 917 days (range 330-2,092 days) after allo-HSCT. OS rate at 1 year was 27% (95% confidence interval [CI], 7-54%) in patients in Day 0-RD+ group, which was significantly lower than those in Day 0-RD− group (73% [95%CI 61-82%], $P<0.001$, **Figure 2A**). In univariate analyses,

Table 1. Patient characteristics

Variables		Day 0-RD−			Day 0-RD+	*p-value
		Pre-conditioning CR (N=44)	Pre-conditioning nCR (N=27)	Total (N=71)	Pre-conditioning nCR (N=11)	
Median age (range) [year-old]		42.5 (17-65)	52 (18-66)	46 (17-66)	50 (26-67)	0.13
Sex	male	23	19	42	10	0.05
	female	21	8	29	1	
Donor-recipient sex	female to male	4	9	13	6	0.02
	other combinations	40	18	58	5	
Disease	AML	42	14	56	9	1.00
	MDS-EB	2	13	15	2	
BM assessment before conditioning	CR	44	0	44	0	<0.001
	nCR	0	27	27	11	
Time interval from BM assessment before conditioning to stem cell infusion (range) [days]		14 (7-48)	15 (6-37)	14 (6-48)	9 (7-32)	0.29
HCT-CI	0 or 1	36	16	52	7	0.49
	≥2	8	11	19	4	
ABO blood type	matched	18	13	31	3	0.35
	mismatched	26	14	40	8	
HLA disparity	8/8 matched BM/PB	28	15	43	6	0.82
	mismatched BM/PB	5	10	15	2	
	≥4/6 matched CB	11	2	13	3	
Stem cell source	related-BM	1	2	3	0	0.90
	related-PB	5	5	10	2	
	unrelated-BM	25	16	41	6	
	unrelated-PB	2	2	4	0	
	unrelated-CB	11	2	13	3	
Conditioning	MAC	29	11	40	5	0.53
	RIC	15	16	31	6	
GvHD prophylaxis	CsA+MTX	6	5	11	2	1.00
	Tac+MTX	38	20	58	9	
	others	0	2	2	0	

AML, acute myeloid leukemia; MDS-EB, myelodysplastic syndrome with excess blats; BM, bone marrow; CB, cord blood; CsA, cyclosporine A; GvHD, graft-versus-host disease; nCR, not in complete remission; HLA, human leukocyte antigen; MTX, methotrexate; PB, peripheral blood; RD, residual disease; RIC, reduced-intensity conditioning; Tac, tacrolimus.
* Comparing total Day 0-RD− with Day 0-RD+ groups.

recipient age at transplantation of older than 45 years old, RIC, and Day 0-RD+ were extracted as risk factors for OS. By multivariate analysis, Day 0-RD+ was identified as an independent risk factor for OS (**Table 2**). Furthermore, when limited to patients with nCR marrow before conditioning, patients without residual blasts in day 0 marrow (Pre-conditioning nCR/Day 0-RD− group) provided significantly better OS compared with Day 0-RD+ group (70% [95% CI, 49-84] and 27% [95% CI, 6.5-54] at 1 year after allo-HSCT, respectively, $P<0.01$), which was comparable with those who maintained CR before conditioning (**Figure 2B**). Median time to relapse after allo-HSCT was shorter in the Day 0-RD+ group (55 days, range of 0-309 days, n=7) than in the Day 0-RD− group (260 days, range of 92-1,057 days, n=16). The cumulative incidences of relapse at 1 year were 46% (95%CI, 14-73%) in patients in Day 0-RD+ group, which was significantly higher than those in Day 0-RD− group (9% [95%CI 3-16%], $P=0.006$, **Figure 2C**). Of note, there was no significant difference in one-year relapse rate between the patients with CR at the point of pre-conditioning assessment (9.1% [95% CI, 2.8-20]) and those with nCR (7.4% [95% CI, 1.2-21]) when limited to Day 0-RD− cases ($P=0.36$, **Figure 2D**).

By multivariate analyses, Day 0-RD+ status was a significant risk factor for relapse (**Table 2**). There was no significant difference in non-relapse mortality between Day 0-RD− and Day 0-RD+ groups (18% [95% CI, 10-28] and 27% [95% CI, 5.6-56] at 1 year after allo-HSCT, respectively, $P=0.49$).

Discussion

Relapse after allo-HSCT remains an obstacle to long-

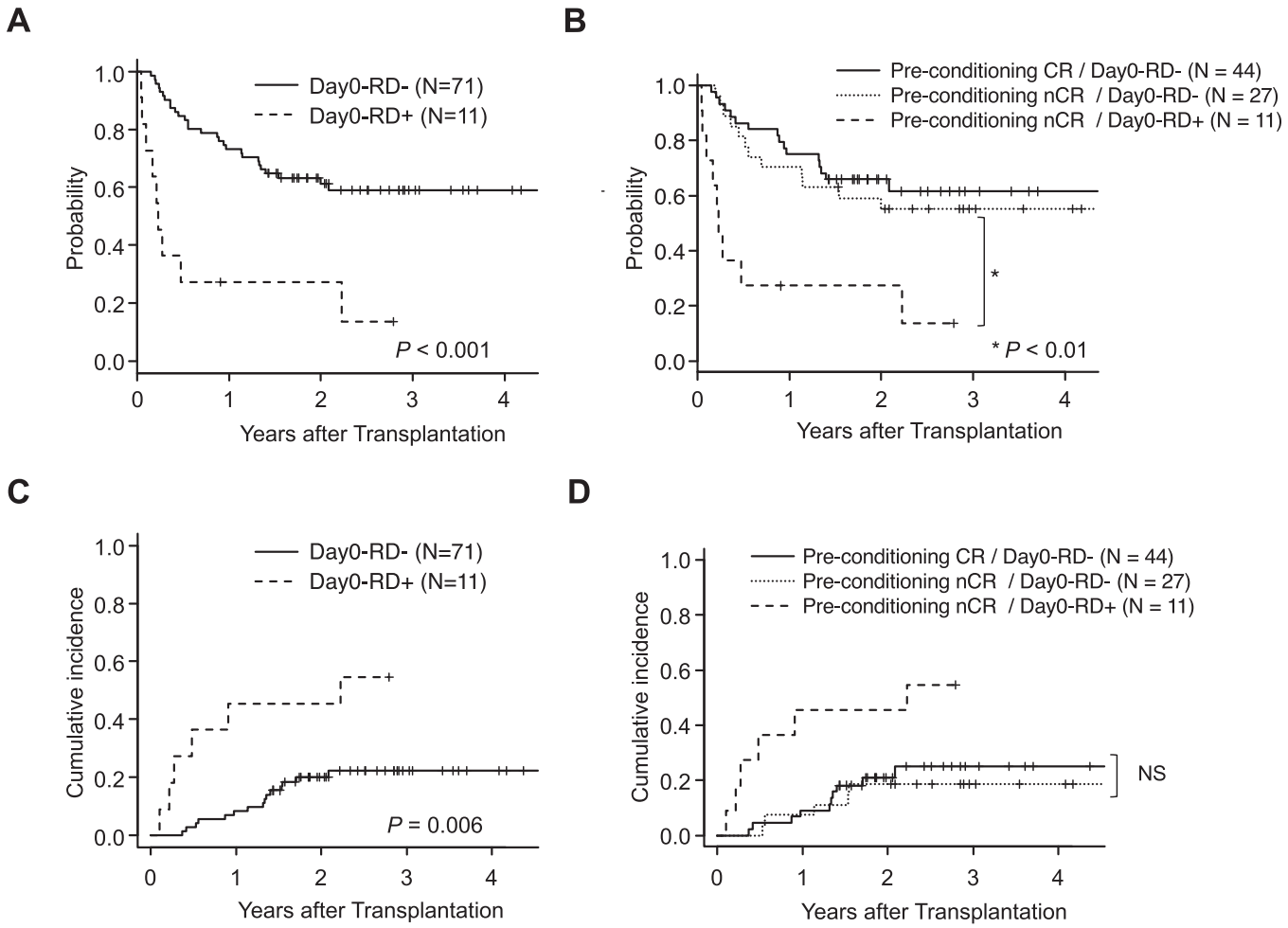

Figure 2. Overall survival and cumulative incidence of relapse according to the status of day 0 marrow
(A) Overall survival according to the status of day 0 marrow. (B) Overall survival according to the status of bone marrow before conditioning (pre-conditioning) and on day 0. (C) Cumulative incidence of relapse according to the status of day 0 marrow. (D) Cumulative incidence of relapse according to the status of bone marrow before conditioning (pre-conditioning) and on day 0. Differences between groups were evaluated by the log-rank test (A and B) and Gray's test (C and D).
NS, not significant.

term survival in myeloid malignancies[12]. Therefore, a novel marker to predict relapse is warranted. In this study, we described the efficacy of evaluating day 0 BM of allo-HSCT after completing a conditioning treatment on predicting OS and relapse, providing a new risk stratification that Day 0-RD+ is an independent risk factor for both OS and relapse, whereas Day 0-RD− represents better OS, even if they were not in CR before conditioning treatment as routinely evaluated. Furthermore, patients who were not in CR at the point of pre-conditioning evaluation showed a comparable prognosis to those with CR before conditioning if they achieved negative RD in the day 0 marrow. Of note, residual blast count of pre-conditioning BM was not significantly associated with that of day 0 marrow, suggesting that the status of day 0 BM more specifically predicts the outcomes after allo-HSCT than disease status assessed before conditioning.

In previous reports, the presence of residual AML cells detected by flow cytometry before RIC was associated with significantly increased relapse, shorter disease-free survival, and poorer OS after allo-HSCT, indicating achieving flow cytometric CR is critical to achieve before conditioning treatment[13]. As the cellularity of day 0 marrow is severely hypoplastic and insufficient to perform flow cytometry to analyze their residual diseases (**Figure 1A**), we used immunohistochemistry of BM clot sections to detect residual blasts in the present study. In this study, minor residual blasts were easily detected even in severely hypoplastic marrow using immunostaining of case-specific blast markers (**Figure 1**). With this method, residual blasts in BM can be assessed faster and at lower costs than quantitative PCR and next-generation sequencing[8,14]. Furthermore, this technique can easily be performed in standard hematopoietic stem cell transplantation hospitals. On the other hand, the main limitation of this study is that the detection sensitivity of our method was not validated. Although the existence of histopathological residual diseases in day 0 marrow was shown to

Table 2. Multivariate analyses for overall survival and cumulative incidence of relapse

Variables	Adverse factors	Overall survival				Cumulative incidence of relapse			
		Univariate	Multivariate			Univariate	Multivariate		
		p-value	HR	(95%CI)	p-value[a]	p-value	HR	(95%CI)	p-value[b]
Recipient age	>45 years old	<0.001	3.1	(1.1-8.6)	0.03	0.05	2.3	(0.90-5.7)	0.08
Donor-recipient sex	female donor to male recipient	0.55	—	—	—	0.67	—	—	—
Disease	AML	0.83	—	—	—	0.22	—	—	—
BM before conditioning	nCR	0.11	—	—	—	0.83	—	—	—
Conditioning	RIC	0.001	1.1	(0.45-2.8)	0.79	0.32	—	—	—
HCT-CI	≥2	0.37	—	—	—	0.20	—	—	—
ABO blood type	Matched	0.22	—	—	—	0.04	3.1	(1.3-7.5)	0.01
HLA disparity	8/8 matched	0.90	—	—	—	0.74	—	—	—
Stem cell source	CB	0.56	—	—	—	0.81	—	—	—
GvHD prophylaxis	Tac-based	0.52	—	—	—	0.13	—	—	
Day 0 marrow	RD (+)	<0.001	3.7	(1.7-8.1)	<0.001	0.006	4.5	(1.8-11)	0.001

HR, hazard ratio; CI, confidence interval; BM, bone marrow; nCR, not in complete remission; RIC, reduced-intensity conditioning; HCT-CI, hematopoietic cell transplantation specific comorbidity index; HLA, human leukocyte antigen; CB, cord blood; GvHD, graft-versus-host disease; Tac, tacrolimus; RD (+), positive residual disease.

[a] All variables with $P<0.1$ from univariate analyses were included in the multivariate analyses performed with the Cox proportional hazard regression model.

[b] All variables with $P<0.1$ from univariate analyses were included in the multivariate analyses performed with the Fine-Gray competing risks regression model.

be a poor prognostic marker in our cohort, BM clot sections are supposed to have histological heterogeneity. Furthermore, the other limitation which may have impacted our findings is that other risk factors of primary diseases such as adverse cytogenetics and genetic alterations were not available for our prognostic analysis[15,16]. Combined with more sensitive methodologies such as quantitative PCR and next-generation sequencing, more significant and clearer impact of day 0 marrow status on outcomes post allo-HSCT could be described in hematological malignancies.

This is a retrospective analysis in a single institution where day 0 BM was routinely examined. However, BM aspiration on the day of allo-HSCT is not widely performed in clinical practice. The clinical significance of minimal/measurable RD has been reportedly assessed in BM before conditioning treatment[8-10]. Because all the patients in CR before conditioning treatment showed Day 0-RD− as expected, our results suggest that Day 0 BM evaluation is necessary for only patients in nCR at the point of pre-conditioning assessment to minimize the risk of this invasive procedure.

Regarding the intensity of conditioning, MAC rather than RIC reportedly reduced the incidence of relapse and improved disease-free survival and OS rate after allo-HSCT in patients with residual leukemia[17,18]. In our analysis, the intensity of conditioning was not significantly associated with the status of RD on day 0 or outcomes including OS and relapse rate by multivariate analyses. In addition, especially in MDS-EB, blast percentage at pre-conditioning was not associated with day 0 BM status. Therefore, it can be speculated that day 0 BM status reflects sensitivity to conditioning treatment.

In the setting of allo-HSCT, disease relapse can occur when graft-versus-leukemia effects are insufficient to suppress the residual disease[19]. In the Day 0-RD+ group, one patient achieved as long as 684 days of disease-free survival by controlling immunosuppressants and chronic graft-versus-host-disease. Our results suggest that day 0 BM assessment carries the potential to prevent relapse in patients with Day 0-RD+ status by early intervention to intensify graft-versus-leukemia effects, such as rapid withdrawal of immunosuppressants or donor lymphocyte infusions[12,20-22].

In summary, we demonstrated the efficacy of immunostaining using blast-specific markers to evaluate residual diseases on day 0 BM of allo-HSCT for the first time. This was a retrospective study including a limited number of patients at a single institution. Therefore, a study in a larger cohort is warranted to confirm the results. Nevertheless, our analyses suggest the detection of Day 0-RD status is a novel prognostic factor that can clinically contribute to constructing post-transplant strategies for improving outcomes in myeloid malignancies.

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Author Contributions

The study concept and design were developed by TS, NK, KM, and MI. Clinical data collection was performed by TS, NK, and YK. Bone marrow specimens were pathologically assessed by MI. Data analyses and manuscript drafting were done by TS and NK. Discussions involved all authors. Administrative and material support was provided by KM and MI. This study was supervised by MI and KM.

Conflicts of Interest

The authors declare no coflict of interest. Disclosure forms provided by the authors are available here.

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