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ORIGINAL ARTICLE

Steep neutrophil recovery following unrelated bone marrow transplantation is a major risk factor for the development of acute graft-vs-host disease—a retrospective study

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SUMMARY

The speed of neutrophil recovery following allogeneic hematopoietic cell transplantation (allo-HCT) varies widely among patients. We retrospectively evaluated the slope of neutrophil recovery (N slope) in 120 patients who underwent a first unrelated bone marrow transplantation with granulocyte-colony-stimulating factor support between 2009 and 2018. The median N slope was 205.5/μl/day. We classified patients into low (n = 59) and high (n = 61) N slope groups with a cutoff value of 200/μl/day. The high N slope group correlated with older patients, RIC regimen, high CD34+ cells, and recent transplantation. The cumulative incidence of grade II–IV acute graft-versus-host disease (aGVHD) was significantly higher in the high N slope group than in the low N slope group (44.3% vs. 16.9%, $P < 0.001$). In multivariate analysis, high N slope was identified as a significant independent risk factor for grade II–IV aGVHD, irrespective of the involved organs. There were no differences in relapse, nonrelapse mortality, or overall survival between the two groups. In conclusion, the difference in N slope after allo-HCT may predict the risk of aGVHD. Prevention and treatment of GVHD according to the changes in the neutrophil count may improve post-transplant complications.

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Key words
acute graft-versus-host disease, allogeneic hematopoietic cell transplantation, neutrophil recovery

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Introduction

Graft-versus-host disease (GVHD) remains a significant complication and a major limitation of successful allogeneic hematopoietic cell transplantation (allo-HCT) [1,2]. The current concept of GVHD development is that antigen-presenting cells activate donor-derived T cells [3] in both lymphoid organs and target tissue [4], and then the donor T cells expand and attack the recipient's tissues [5]. On the other hand, according to previous reports, in the early phase of GVHD before the expansion of alloreactive cytotoxic T cells, a conditioning regimen with irradiation or chemotherapy leads to the activation of myeloid cells [6–9], and neutrophils

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are involved in the onset and exacerbation of GVHD [7].

The speed of neutrophil recovery following allo-HCT varies widely among patients. Some patients have a rapid increase in neutrophils and early engraftment, whereas others have a slow increase after the appearance of neutrophils and take time to achieve engraftment. It remains unclear whether this difference affects the development of GVHD. Therefore, we retrospectively analyzed the influence of the slope of neutrophil recovery following allo-HCT on post-transplant complications and prognosis.

Methods

Patients

This retrospective study included adult recipients (>17 years) with acute leukemia, myelodysplastic syndromes (MDS), chronic myeloid leukemia (CML), lymphoma (ML), aplastic anemia (AA), adult T-cell leukemia/lymphoma, myelofibrosis (MF), chronic myelomonocytic leukemia (CMML), or multiple myeloma (MM) who underwent a first unrelated bone marrow transplantation at Jichi Medical University between January 2009 and December 2018. Patients who failed to achieve engraftment or did not receive granulocyte-colony-stimulating factor (G-CSF) were excluded. Finally, 120 recipients were analyzed. The median duration of follow-up in survivors was 890.5 days. Clinical data were obtained from individual medical records. This analysis was approved by the Ethics Committee of Jichi Medical University.

Conditioning regimens and immunosuppressive agents

Conditioning regimens were classified as myeloablative conditioning regimens (MAC) or reduced-intensity conditioning regimens (RIC) based on a previous report [10]. Briefly, conditioning regimens that included total body irradiation (TBI) >8 Gy, melphalan >140 mg/m^2, or oral busulfan (BU) >8 mg/kg (>6.4 mg/kg i.v.) were classified as MAC. Other regimens were classified as RIC. The most frequently used MAC was a combination of cyclophosphamide and either TBI 12 Gy or BU (3.2 mg/kg i.v. once daily for 4 days). RIC mainly consisted of fludarabine-based regimens, such as fludarabine combined with BU or melphalan. GVHD prophylaxis was provided by continuous infusion of tacrolimus (TAC) combined with short-term methotrexate (MTX; 10–15 mg/m^2 on day 1, 7–10 mg/m^2 on days 3 and 6, and an optional dose on day 11). The dose of TAC was adjusted to maintain a blood concentration between 12 and 15 ng/ml. Anti-thymocyte globulin (ATG) was administered at 1.25 mg/kg per day on days 4 and 3 in some cases of human leukocyte antigen (HLA) 1-locus mismatched transplantation. G-CSF for accelerating bone marrow recovery was started on 7 days after allo-HCT in all patients, except two patients whose G-CSFs were started on day 1. Lenograstim (5 μg/m^2) or filgrastim (300 μg/m^2) were used.

Definitions

The slope of neutrophil recovery (N slope) following allo-HCT was defined as the increase in neutrophil count from the last day of the lowest neutrophil count after transplantation to the day of neutrophil engraftment. Neutrophil engraftment was defined as the first of three consecutive days on which the patient had an absolute neutrophil count of 0.5×10^3/μl or higher. Standard-risk diseases included acute leukemia in first or second complete remission (CR), CML in the first or second chronic phase, lymphoma in CR, MDS, AA, MF, CMML, and MM. Other diseases were classified as high risk. Acute GVHD (aGVHD) was diagnosed and graded according to established criteria [11].

Statistical analysis

We evaluated the predictive value of the N slope for aGVHD using the area under the receiver operating characteristic (ROC) curve and determined the cutoff value to maximize the sum of sensitivity and specificity. Patients were divided into two groups according to this cutoff value. Correlations between the N slope level and various clinic pathological characteristics were assessed by Fisher's exact test for categorical variables and the Mann–Whitney *U* test for continuous variables. Incidences of clinical events were estimated by cumulative incidence analysis and compared by Gray's test. Competing events were death without each event, disease relapse for NRM, and disease relapse and NRM for GVHD. Overall survival (OS) probabilities were estimated by the Kaplan–Meier method and compared by the log-rank test. Fine and Gray's proportional hazard regression model for cumulative incidence and the Cox proportional hazards regression model for OS were used for multivariate analyses. Factors that showed borderline significance ($P < 0.1$) in univariate analyses were subjected to multivariate analysis. Bootstrap validation was

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performed using validate function of the rms package. All statistical analyses were performed using EZR version 1.37 (Saitama Medical Center, Jichi Medical University, http://www.jichi.ac.jp/saitama-sct/SaitamaHP.files/statmedEN.html), which is a graphical user interface for R [12].

Results

Neutrophil slope

The median N slope was 205.5/μl/day (range, 26.4–7574). ROC analysis showed that the cutoff value of N slope for grade II–IV aGVHD was 207.5/μl/day [sensitivity 0.73; specificity 0.6; area under the curve (AUC), 0.67; 95% confidence interval (CI), 0.59–0.79], which was close to the median value. We then classified patients into low and high N slope groups according to the cutoff value of 200/μl/day.

Patient characteristics

Of the 120 recipients, 61 (51%) and 59 (49%) were classified into the high and low N slope groups, respectively. The patients' characteristics are summarized in Table 1. Compared with the low N slope group, patients in the high N slope group were older ($P = 0.03$), less likely to have received day 11 MTX ($P = 0.004$), more likely to have received a RIC regimen ($P = 0.03$), more likely to have received $\geq 1.5 \times 10^6$/kg infused CD34+ cells ($P = 0.04$), and more likely to have undergone a first transplantation in the period between 2009 and 2013 ($P = 0.03$). Engraftment syndrome (ES) was observed in nine recipients. There was no significant difference in the cumulative incidence of ES at 30 days after allo-HCT between high and low N slope groups (8.2% vs. 5.1%, $P = 0.75$).

Acute graft-versus-host disease

Of the 120 recipients, 37 experienced grade II–IV aGVHD, with a cumulative incidence of 31% (95% CI, 23–38%) at 100 days after allo-HCT. The cumulative incidence of aGVHD was higher in the high N slope group (44%; 95% CI, 32–56%) than in the low N slope group (17%; 95% CI, 9–28%; $P < 0.001$; Fig. 1a). With regard to the risk factors for aGVHD, male sex, donor age ≥40 years, TBI (any dose), high N slope, and first transplantation between 2009 and 2013 were significant in a univariate analysis. In a multivariate analysis, donor age ≥40 years [hazard ratio (HR) 3.63, $P = 0.003$], ABO major mismatch (HR 3.05, $P = 0.003$), TBI (HR 2.83, $P = 0.02$), high N slope (HR 3.73, $P < 0.001$), and first transplantation between 2009 and 2013 (HR 3.28, $P = 0.006$) were identified as independent significant risk factors (Table 2). When the above seven factors by univariate analysis were applied to the stepwise selection, high N slope remained to be a significant factor for grade II–IV aGVHD (HR 2.78, $P < 0.001$), together with donor age ≥40 years (HR 3.91, $P < 0.001$) and first transplantation between 2009 and 2013 (HR 3.32, $P < 0.001$). The cutoff value based on ROC analysis might have resulted in arbitrary grouping, and we validated the results using bootstrap resampling. The original and the corrected concordance indices were 0.647 and 0.643, respectively, and therefore, the problem of optimism seemed negligible.

Grade III–IV aGVHD was observed in nine recipients, with a cumulative incidence of 7.5% (95% CI, 3.7–13.1%) at 100 days after allo-HCT. There was no significant difference in the cumulative incidence of grade III–IV aGVHD between the high and low N slope groups (9.8%, 95% CI, 4–19% vs. 5.1%, 95% CI, 1.3–13%, $P = 0.33$; Fig. 1b).

Grade I and II aGVHD was observed in 36 and 28 recipients, respectively. There was no significant difference in the cumulative incidence of grade I aGVHD at 100 days after allo-HCT between two groups (26.2% vs. 33.9%, $P = 0.44$), but the cumulative incidence of grade II aGVHD was higher in the high N slope group than in the low N slope group (34.4% vs. 11.9%, $P = 0.002$; Fig. S1).

Target organs of aGVHD

Of the 120 recipients, 21 experienced gut aGVHD (stage 1–4), with a cumulative incidence of 18% (95% CI, 12–26%) at 100 days after allo-HCT. The cumulative incidence of gut aGVHD was higher in the high N slope group (26%; 95% CI, 15–38%) than in the low N slope group (10%; 95% CI, 4.2–20%; $P = 0.02$; Fig. 2a). Three recipients experienced liver aGVHD (stage 1–4), with a cumulative incidence of 3.6% (95% CI, 1.2–8.4%) at 100 days after allo-HCT. The cumulative incidence of liver aGVHD was higher in the high N slope group (7.5%; 95% CI, 2.4–17%) than in the low N slope group (0%; 95% CI, 0.0–0.0%; $P = 0.04$; Fig. 2b). Nineteen recipients experienced severe skin aGVHD (stage 3–4), with a cumulative incidence of 16% (95% CI, 10–24%) at 100 days after allo-HCT. The cumulative incidence of severe skin aGVHD in the high N slope group (23%; 95% CI, 13–34%) tended to be higher than that in the low N slope group (10%; 95% CI, 4.1–20%; $P = 0.07$; Fig. 2c).

Table 1. Patient characteristics according to the N slope

	Total $n = 120$	Low N slope $n = 59$	High N slope $n = 61$	P
Age, years, median (range)	50 (18–69)	45 (19–68)	52 (18–69)	0.03
Sex				
Male	77 (64.2%)	40 (67.8%)	37 (60.7%)	0.45
Female	43 (35.8%)	19 (32.2%)	24 (39.3%)	
Sex mismatch				
Female to male	17 (14.2%)	12 (20.3%)	5 (8.2%)	0.07
Other	103 (85.8%)	47 (79.7%)	56 (91.8%)	
Disease				
AML	48 (40%)	24 (40.7%)	24 (39.3%)	0.69
ALL	27 (22.5%)	16 (27.1%)	11 (18%)	
MDS	20 (16.7%)	9 (15.3%)	11 (18%)	
CML	4 (3.3%)	2 (3.4%)	2 (3.3%)	
ML	9 (7.5%)	4 (6.8%)	5 (8.2%)	
AA	3 (2.5%)	0 (0%)	3 (4.9%)	
Other	9(7.5%)	4 (6.8%)	5 (8.2%)	
Disease risk				
High	18 (15%)	8 (13.6%)	10 (16.4%)	0.80
Standard	102 (85%)	51 (86.4%)	51 (83.6%)	
HLA matching*				
Match	84 (70%)	45 (76.3%)	39 (63.9%)	0.17
Mismatch	36 (30%)	14 (23.7)	22 (36.1%)	
Donor age				
<40 years	65 (54.2%)	33 (55.9%)	32 (52.5%)	0.72
≥40 years	55 (45.8%)	26 (44.1%)	29 (47.5%)	
Day-11 MTX				
Yes	65 (54.2%)	40 (67.8%)	25 (41%)	0.004
No	55 (45.8%)	19 (32.2%)	36 (59%)	
ATG				
Yes	16 (13.3%)	7 (11.9%)	9 (14.8%)	0.79
No	104 (86.7%)	52 (88.1%)	52 (85.2%)	
Conditioning				
MAC	100 (83.3%)	54 (91.5%)	46 (75.4%)	0.03
RIC	20 (16.7%)	5 (8.5%)	15 (24.6%)	
TBI regimen				
Yes	78 (65%)	35 (59.3%)	43 (70.5%)	0.25
No	42 (35%)	24 (40.7%)	18 (29.5%)	
Infuse CD34+ cells				
$<1.5 \times 10^6$/kg	50 (44.6%)	30 (55.6%)	20 (34.5%)	0.04
$\geq 1.5 \times 10^6$/kg	62 (55.4%)	24 (44.4%)	38 (65.5%)	
Transplantation year				
2009–2013	45 (37.5%)	16 (27.1%)	29 (47.5%)	0.03
2014–2018	75 (62.5%)	43 (72.9%)	32 (52.5%)	

AA, aplastic anemia; ALL, acute lymphoblastic leukemia; AML, acute myeloid leukemia; ATG, anti-thymocyte globulin; CML, chronic myeloid leukemia; HLA, human leukocyte antigen; MAC, myeloablative conditioning; MDS, myelodysplastic syndrome; ML, malignant, lymphoma; RIC, reduced-intensity conditioning; TBI, total body irradiation.

*HLA mismatch indicates antigen mismatch at the HLA-A, B, C, or DR loci.

Survival

Of the 120 patients, 40 died, including 16 deaths due to nonrelapse complications, during the follow-up period (median 29 months, range 1–116 months). The N slope was not associated with the cumulative incidences of NRM ($P = 0.32$), relapse ($P = 0.76$), and OS ($P = 0.65$; Fig. 3).

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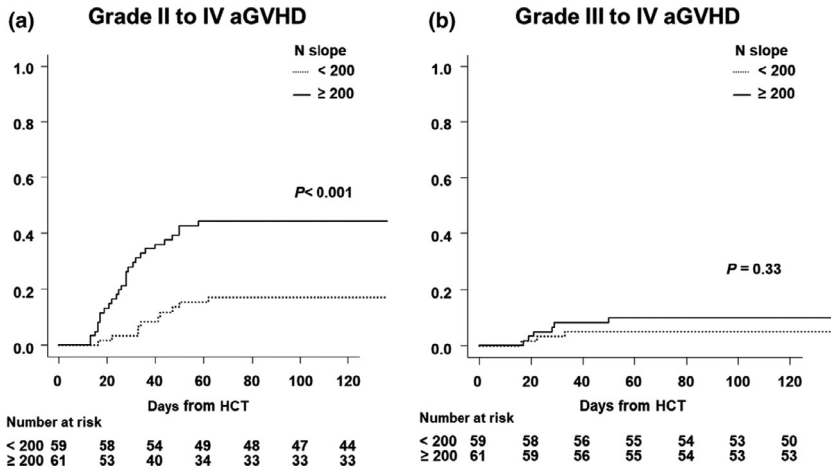

Figure 1 Cumulative incidences of (a) grade II–IV and (b) grade III–IV acute graft-versus-host disease (aGVHD) according to slope of neutrophil recovery (N slope).

Discussion

This retrospective study showed that a relatively high rate of neutrophil recovery following allo-HCT was associated with a higher incidence of grade II–IV aGVHD, regardless of the involved organs.

A steep neutrophil recovery was reported to be a risk factor for the development of ES [13], and there are many overlaps between aGVHD and ES in clinical and pathophysiologic features. In this study, there was no significant difference about the incidence of ES between the high and low N slope groups. However, it might be due to a small number of patients who were diagnosed with ES by strictly defined criteria. Among patients who developed aGVHD in this study, high N slope group tended to develop aGVHD relatively earlier after engraftment compared with the low N slope group. Further studies are needed to determine whether aGVHD and/or steep neutrophil recovery is in any way related to the pathogenesis of ES.

In terms of the effect of neutrophil engraftment on the development of GVHD, several studies showed that the use of G-CSF after allo-HCT was an independent risk factor for the development of aGVHD [14,15]. G-CSF after allo-HCT might impact immune effector cells as well as stem cells or neutrophils, since it could adjust the production of inflammatory cytokines and promote the mobilization of T helper 2-inducing dendritic cells and immune polarization [16–19]. The wide range of effectors that can be induced by G-CSF might affect each phase of the pathophysiology of aGVHD. In this study, which used G-CSF in all cases, a high N slope may show high reactivity to G-CSF, reflecting the activation of immune effector cells related to the onset of GVHD.

Total body irradiation is a risk factor for mucositis and gastroenteritis after allo-HCT, both of which may have roles in accelerating GVHD [20,21]. The TBI regimen was more frequently used in patients in the high N slope group, but the multivariate analysis demonstrated that rapid neutrophil recovery (e.g., high N slope) was a risk factor for the development of aGVHD independent of TBI. Furthermore, TBI was not associated with the development of gut aGVHD among those in the high N slope group. Taken together, these results suggest that TBI was not directly associated with the development of aGVHD.

A high N slope was associated with older age, a lower frequency of administration of MTX on day 11, use of a RIC regimen and higher numbers of infused CD34+ cells. These factors can be classified into those associated with infused donor stem cells and those associated with the recipient's bone marrow environment. The number of infused CD34+ stem cells and ongoing administration of MTX through day 11 may each have a direct impact on neutrophil recovery. On the other hand, the MAC regimen may alter the bone marrow microenvironment [22], ultimately resulting in a more gradual neutrophil recovery.

This was the first clinical report showing an association between neutrophil recovery and the onset of GVHD. Neutrophils recruited into tissues upon bacterial invasion cause tissue injury and exacerbate GVHD [7], although the association between difference in neutrophil recovery and neutrophil tissue damage was unknown in this study. Further research is required, but if rapid neutrophil recovery is associated with the severity of neutrophil induced tissue damage, JAK2 inhibition related to G-CSF signaling may be useful in reducing GVHD.

Table 2. Factors for grade II–IV acute GVHD in univariate and multivariate analyses

	n	Cumulative incidence of aGVHD II–IV at day + 100 (95% CI), %	Univariate *P*	HR (95% CI)	Multivariate *P*
Recipient age					
<60	102	31.4 (22.6–40.5)	0.68		
≥60	18	27.8 (9.6–49.6)			
Donor age					
<40	65	18.5 (10.1–28.8)	0.002	1	Reference
≥40	55	45.5 (31.9–58.1)		3.63 (1.57–8.40)	0.003
Sex					
Male	77	23.4 (14.6–33.3)	0.02	1	Reference
Female	43	44.2 (28.9–58.4)		0.56 (0.27–1.18)	0.13
Disease					
Acute leukemia	76	34.2 (23.7–44.9)	0.27		
Other	44	25 (13.3–38.5)			
Disease risk					
High	18	44.4 (20.8–65.8)	0.16		
Standard	102	28.4 (20–37.4)			
ABO mismatch					
Major mismatch	27	44.4 (25.1–62.2)	0.06	3.05 (1.47–6.31)	0.003
Other	93	26.9 (18.3–36.2)		1	Reference
Conditioning regimen					
MAC	100	32 (23.1–41.3)	0.48		
RIC	20	25 (8.8–45.4)			
HLA matching*					
Match	84	28.6 (19.3–38.5)	0.47		
Mismatch	36	36.1 (20.8–51.7)			
TBI					
Yes	78	38.5 (27.7–49.1)	0.01	2.83 (1.20–6.67)	0.02
No	42	16.7 (7.2–29.4)		1	Reference
Day-11 MTX					
Yes	65	24.6 (14.9–35.6)	0.08	1	Reference
No	55	38.2 (25.4–50.9)		1.78 (0.82–3.88)	0.15
ATG					
Yes	16	18.8 (4.3–41)	0.26		
No	104	32.7 (23.9–41.8)			
Engraftment day					
<18	63	36.5 (24.7–48.3)	0.12		
≥18	57	24.6 (14.3–36.3)			
N slope					
<200	59	16.9 (8.7–27.6)	<0.001	1	Reference
≥200	61	44.3 (31.5–56.3)		3.73 (1.88–7.42)	<0.001
Infused CD34+ cells					
$<1.5 \times 10^6$/kg	50	30 (17.9–43)	0.81		
$\geq 1.5 \times 10^6$/kg	62	32.2 (21–44)			
Transplantation year					
2009–2013	45	46.7 (31.5–60.5)	0.003	3.28 (1.40–7.68)	0.006
2014–2018	75	21.3 (12.9–31.2)		1	Reference

ATG, anti-thymocyte globulin; MAC, myeloablative conditioning; RIC, reduced-intensity conditioning; TBI, total body irradiation.

*HLA mismatch indicates antigen mismatch at the HLA-A, B, C, or DR loci.

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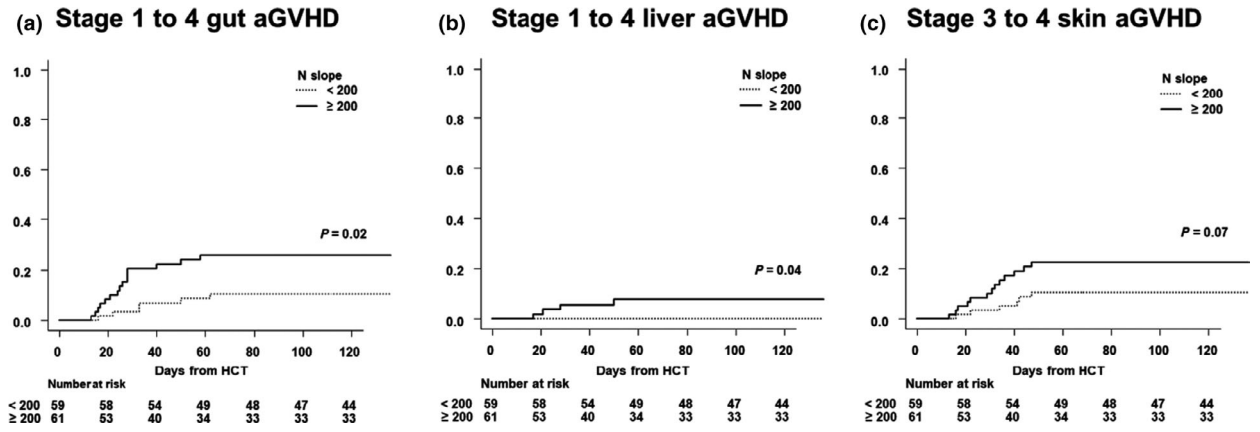

Figure 2 Cumulative incidences of (a) stage 1–4 gut, (b) stage 1–4 liver, and (c) stage 3–4 skin acute graft-versus-host disease (aGVHD).

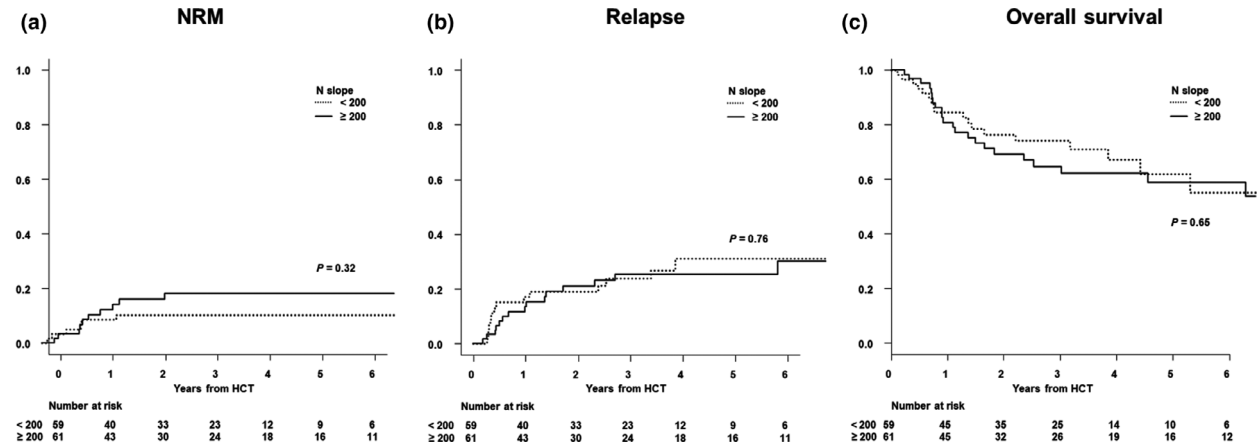

Figure 3 Cumulative incidences of nonrelapse mortality (NRM) (a) and relapse (b) according to slope of neutrophil recovery (N slope). Probability of overall survival (OS) (c) according to N slope.

This study has some limitations. For example, this study was retrospective, and included patients with heterogeneous characteristics and diagnoses. The statistical power of our analyses was limited, leading to the possibility that some potentially significant factors might have been missed. Furthermore, the N slope cutoff value (200/μl/day) was determined from the approximate values of both the median N slope (205.5/μl/day) for all patients and for patients with grade II–IV aGVHD defined by the ROC curve (207.5/μl/day). Therefore, this cutoff value may not be appropriate other events such as transplant-related complications and mortality other than those directly related to aGVHD. Third, transplantation between 2009 and 2013, the first half of the period of this study, resulted in a significant increase in aGVHD. As the possible reason for this, ATG was less used in HLA 1-locus mismatched transplantation in the period (4.7% vs. 23.0 %, $P = 0.03$). In addition, the rate of TBI regimen, detected as a risk of aGVHD in this study, was lower in the later period transplantation (82.2% vs. 52.0%, $P < 0.001$). Finally, of the 25 patients diagnosed with aGVHD by biopsy, seven demonstrated neutrophil infiltration into peripheral tissues (five in the high N slope group and two in the low N slope group). As such, our results could not demonstrate a clear association between neutrophil infiltration and a diagnosis of aGVHD.

In summary, a rapid rate of neutrophil recovery following allo-HCT was associated with an increased risk for the development of aGVHD, although there was no similar impact on the incidence of relapse, NRM, or OS. Prevention and treatment of GVHD according to changes in the neutrophil count may improve post-transplant complications.

Authorship

TN: participated in research design, data analysis, and the writing of the paper and approved the final manuscript. SF: participated in research design, data

analysis, and the writing of the paper and approved the final manuscript. TI: read and approved the final manuscript. SK: read and approved the final manuscript. YT: read and approved the final manuscript. SI: read and approved the final manuscript. SO: read and approved the final manuscript. KM: read and approved the final manuscript. KU: read and approved the final manuscript. DM: read and approved the final manuscript. HN: read and approved the final manuscript. RY: read and approved the final manuscript. KM: read and approved the final manuscript. YK: read and approved the final manuscript. CY: read and approved the final manuscript. MA: read and approved the final manuscript. KH: read and approved the final manuscript. KS: read and approved the final manuscript. IO: read and approved the final manuscript. KO: read and approved the final manuscript. KM: read and approved the final manuscript. YK: participated in research design, data analysis, and the writing of the paper and approved the final manuscript.

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Conflicts of interest

The authors declare no conflicts of interest.

SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section at the end of the article.

Figure S1. Cumulative incidences of (a) grade I and (b) grade II aGVHD according to N slope.

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