Blood Cells, Molecules and Diseases 74 (2019) 5–12

Contents lists available at ScienceDirect

Blood Cells, Molecules and Diseases

journal homepage: www.elsevier.com/locate/bcmd

# Plasma-based protein biomarkers can predict the risk of acute graft-versus-host disease and non-relapse mortality in patients undergoing allogeneic hematopoietic stem cell transplantation

Junghoon Shin[a,b,1], Kisoon Dan[c,1], Dohyun Han[c], Ji-Won Kim[d], Kyung Kon Kim[e], Youngil Koh[a,b], Dong-Yeop Shin[a,b], Junshik Hong[a,b], Sung-Soo Yoon[a,b], Seonyang Park[a], Sang Hoon Song[f,*], Inho Kim[a,b,**]

[a] Department of Internal Medicine, Seoul National University Hospital, Seoul, Republic of Korea
[b] Cancer Research Institute, Seoul National University College of Medicine, Seoul, Republic of Korea
[c] Proteomics Core Facility, Biomedical Research Institute, Seoul National University Hospital, Seoul, Republic of Korea
[d] Department of Internal Medicine, Seoul National University Bundang Hospital, Seongnam, Republic of Korea
[e] Department of Convergence Medicine, Asan Institute for Life Sciences, Asan Medical Center, Seoul, Republic of Korea
[f] Department of Laboratory Medicine, Seoul National University Hospital, Seoul, Republic of Korea

ARTICLE INFO

Editor: Mohandas Narla

*Keywords:*
Biomarker
Acute graft-versus-host disease
Allogeneic hematopoietic stem cell transplantation
Non-relapse mortality
Proteomics

ABSTRACT

Predictive biomarkers for acute graft-versus-host disease (aGVHD) is currently lacking. In this study, we employed an unbiased proteome profiling method to prospectively collected plasma samples from allogeneic hematopoietic stem cell transplantation (alloHSCT) recipients to identify protein biomarkers that predict the risk of aGVHD and non-relapse mortality (NRM). In the discovery set, including five aGVHD patients and five controls, we identified seven candidate proteins. Patients with high levels of these proteins tended to exhibit a higher risk of aGVHD and NRM compared to patients with low levels in post-engraftment plasma samples from an independent validation set ($n$ = 89). Tissue inhibitor of metalloproteinase 1, plastin-2, and regenerating islet-derived protein 3-α were selected as the most-predictive biomarkers via an exhaustive variable screening algorithm and were collectively used to develop a biomarker panel score ranging from 0 to 3. The biomarker panel score correlated significantly with aGVHD and NRM risk in univariable and multivariable Cox models. Furthermore, using the biomarker panel score in conjunction with clinical predictors significantly improved the discriminatory performance of the Cox model in predicting aGVHD and NRM risk. Our findings suggest that plasma-derived protein biomarkers can be used to predict aGVHD and NRM before the onset of clinical manifestations.

## 1. Introduction

Allogeneic hematopoietic stem cell transplantation (alloHSCT) is a cornerstone treatment for many malignant and non-malignant hematologic disorders, often providing the only chance of cure. In malignant diseases such as acute leukemia and lymphoma, alloHSCT provides an additional therapeutic benefit via the graft-versus-tumor effect [1,2]. However, despite routine prophylaxis with immunosuppressive agents, in approximately half of alloHSCT recipients, the allogeneic immune system recognizes and attacks normal host tissue, a condition known as acute graft-versus-host disease (aGVHD) [3]. aGVHD represents a critical barrier to widespread clinical application of alloHSCT as an upfront therapeutic option and is a major cause of non-relapse mortality (NRM) in alloHSCT patients [1].

The current approach to aGVHD prophylaxis is poorly standardized across countries and centers [4]. Therefore, an emphasis on establishing a consensus guideline regarding optimal prevention strategies has emerged [5]. However, most current recommendations depend solely

* Correspondence to: S.H. Song, Department of Laboratory Medicine, Seoul National University Hospital and College of Medicine, 101, Daehak-ro, Jongno-gu, Seoul 03080, Republic of Korea.
** Correspondence to: I. Kim, Department of Internal Medicine, Seoul National University Hospital and College of Medicine, 101, Daehak-ro, Jongno-gu, Seoul 03080, Republic of Korea.
*E-mail addresses:* cloak21@snu.ac.kr (S.H. Song), ihkimmd@snu.ac.kr (I. Kim).
[1] These authors contributed equally to this work.

https://doi.org/10.1016/j.bcmd.2018.10.001
Received 2 September 2018; Accepted 1 October 2018
Available online 04 October 2018

on clinical factors that are insufficient for precise risk stratification, such as conditioning regimen, donor-recipient relationship, and HLA match status [5]. Development of a finely tailored prophylactic therapy based on an individual's risk profile will thus require a more comprehensive model that enables accurate prediction of aGVHD risk before clinical manifestation of symptoms.

Over the last decade, extensive research has been devoted to identifying GVHD-specific biomarkers with diagnostic or prognostic value. The greatest success achieved to date involves plasma-derived protein markers associated with aGVHD diagnosis [6–9], treatment response [7,9–12], and survival [6–12]. Studies have suggested that the proteome patterns of patients who develop aGVHD are distinct from patients who do not develop the disease, even before the onset of clinical symptoms, suggesting that risk-adaptive prophylactic therapies are possible [13–16]. However, although both diagnostic and prognostic biomarkers have been extensively investigated, few studies have explored the value of these biomarkers for predicting future occurrence of aGVHD. A support vector machine-derived classifier based on urine proteome profiles of aGVHD patients was capable of predicting aGVHD occurrence prior to clinical diagnosis with promising sensitivity and specificity [13,14]. However, the extensive filtration, reabsorption, and metabolism proteins undergo during renal excretion suggest that the plasma proteome might better reflect the pathophysiology of aGVHD [17]. Other researchers identified plasma biomarkers that could diagnose aGVHD in the preclinical stage, but they focused only on candidate biomarkers preselected based on biological relevance [15,16]. In this study, we employed an unbiased proteomics approach using a liquid chromatography (LC)-tandem mass spectrometry (MS/MS)-based method to identify protein biomarkers that can predict the risk of aGVHD and NRM in prospectively collected plasma samples from alloHSCT patients. We found that a biomarker panel score involving three proteins significantly correlated with aGVHD and NRM risk and improved the discriminatory performance of the model when added to clinical characteristics in terms of predicting aGVHD and NRM risk before the onset of clinical manifestations.

## 2. Materials and methods

### 2.1. Study design

Eligible patients were retrospectively identified from a registry of patients with benign or malignant hematologic disorders who underwent first alloHSCT between 2005 and 2011 at Seoul National University Hospital (SNUH) and had provided written informed consent for plasma sample collection. All patients in the registry provided plasma samples at the beginning of conditioning chemotherapy, on the day of stem cell infusion, and weekly thereafter until discharge or death. Clinical diagnosis of aGVHD involved histologic confirmation when appropriate. aGVHD was graded according to the modified Glucksberg criteria [18]. Data regarding patient characteristics and transplantation-related outcomes were obtained from medical records.

The study consisted of a discovery phase and a validation phase. In the discovery phase, proteome profiling of plasma samples from 10 patients was conducted to identify candidate protein biomarkers potentially associated with aGVHD development. In the validation phase, the predictive value of the identified candidate biomarkers was evaluated with respect to aGVHD and NRM risk using an independent validation cohort (validation set).

### 2.2. Discovery set

To maximize the number of protein biomarkers identified based on significantly higher levels in aGVHD patients, we selected five patients who developed aGVHD a median of 12 (range, 5–20) days after alloHSCT and five diagnosis- and conditioning-matched controls who never developed aGVHD. These patients served as the discovery set. Day-matched plasma samples obtained a median of 14 (range, 14–22) days after alloHSCT were selected from each patient pair. Plasma of patients who developed aGVHD was obtained a median of 2 (range, 1–10) days after aGVHD onset.

First, we compared the proteome profiles of pooled plasma from the aGVHD-positive and aGVHD-negative groups using a nanoflow LC-MS/MS-based, label-free, quantitative proteomics method. Only proteins exhibiting $\geq$1.5-fold higher level of expression in the pooled plasma of aGVHD-positive patients compared with that of aGVHD-negative patients were subjected to subsequent LC-multiple reaction monitoring (MRM) MS method development for relative quantification. Using this approach, we determined the relative abundance of multiple proteotypic peptides corresponding to each protein in the individual plasma samples. Based on the results, we identified candidate protein biomarkers exhibiting significantly higher relative expression (Wilcoxon rank sum test $P$-value $< 0.2$ for all proteotypic peptides corresponding to a given protein) in the plasma of aGVHD-positive patients compared with that of aGHVD-negative patients. Detailed experimental procedures are provided in the Supplementary methods.

### 2.3. Validation set

To ensure the relevance of candidate proteins as predictive biomarkers and subject homogeneity, the validation set was selected based on the following eligibility criteria: (1) successful engraftment within 28 days of alloHSCT, and (2) no clinical manifestations of aGVHD onset before the engraftment day. Engraftment was defined as the first three consecutive days with an absolute neutrophil count $\geq$500/μL after alloHSCT. Engraftment day was defined as the first day when engraftment was confirmed (the last of the three consecutive days). In total, 91 patients met these criteria. Plasma samples obtained from these patients on engraftment day or later (but not later than the onset of aGVHD) were used for measurement of candidate biomarker levels using the LC-MRM MS procedure for absolute quantification. Two patients who had no plasma samples obtained within this timeframe were excluded, leaving 89 patients in the validation set. As the plasma samples were collected at one-week intervals, most patients had multiple samples fulfilling these criteria. Therefore, plasma obtained on the day nearest to the engraftment day was selected to minimize sampling-day bias.

Two proteotypic peptides corresponding to each candidate protein were quantified using our LC-MRM MS procedure, and the levels of all peptide pairs exhibited a high correlation (Pearson correlation coefficient $> 0.97$; Fig. S1). Therefore, we averaged the levels of the two peptides from each protein to obtain a protein-level value. These values were then logarithmically transformed and dichotomized at the median to classify patients into ‘high’ or ‘low’ level groups.

### 2.4. Statistical analysis

In the validation set, univariable Cox proportional hazards regression analysis for all potential clinical predictors and each of the candidate biomarkers was first performed to assess the relationship of these variables with aGVHD and NRM risk. Next, we used a two-step exhaustive screening procedure to construct optimal multivariable Cox models to predict aGVHD and NRM risk [19]. First, we selected the best subset of clinical covariates to include in the multivariable model. Second, with adjustment for the selected clinical predictors, we identified the best combination of biomarkers to individually predict aGVHD and NRM risk. The optimal model was defined by the lowest Akaike Information Criterion among those models containing all possible combinations of input variables. Using the identified combination of biomarkers, we defined a biomarker panel score as the number of protein markers present at high levels in a given patient. This score was used as a continuous variable to assess the collective effect of biomarker level on aGVHD and NRM risk in subsequent analyses.

The incremental value of model performance was evaluated by

adding the biomarker panel score to the models including only clinical predictors based on the likelihood ratio test, 5-fold cross-validated C (5-CVC) indices, and a continuous form of the survival-based net reclassification improvement (NRI) index [20,21]. To obtain a distribution of 5-CVC indices, we repeated random splitting of the dataset into five groups and calculated 5-CVC 200 times for each model. The Student *t*-test was used to compare the 5-CVC indices between the model including clinical predictors only and that including clinical predictors plus biomarker panel score. The NRI index calculation was based on 6-month Kaplan-Meier estimates of aGVHD risk and 1-year Kaplan-Meier estimates of NRM risk. The percentile bootstrap method was used to estimate 95% confidence intervals (CIs) and determine the statistical significance of the NRI index. As a supplementary analysis, we also assessed the predictive value of individual candidate biomarkers independent of clinical characteristics using the same variable selection algorithm described above.

Because differences in cause-specific risk may not translate into differences in actual incidence [22], we estimated the cumulative incidence of aGVHD and NRM using a cumulative incidence function and compared the results between groups using Gray's test [23]. Death without aGVHD and relapse were considered competing events for aGVHD and NRM, respectively. Finally, we constructed a Fine-Gray model to assess whether the biomarker panel score could predict the cumulative incidence of aGVHD and NRM [24].

All tests were two-tailed. A $P$-value of $< 0.2$ was considered indicative of significance in the discovery proteomic analysis, and a $P$-value of $< 0.05$ was considered indicative of significance in the validation set analysis. R software, version 3.5.0 (R Foundation for Statistical Computing, Vienna, Austria), was used for statistical analyses.

### 2.5. Compliance with ethical standards

The study was approved by the Institutional Review Board at SNUH (protocol number: 1306-093-499). All patients or their legal guardians provided written informed consent for plasma sample collection for research purposes. All procedures were carried out in accordance with the Helsinki Declaration (revised 2013; World Medical Association).

## 3. Results

### 3.1. Patient characteristics

Patient characteristics are summarized separately for the discovery and validation sets in Table 1. The discovery set included two acute myeloid leukemia (AML) pairs, one acute lymphoblastic leukemia (ALL) pair, one acute biphenotypic leukemia-ALL pair, and one myelodysplastic syndrome (MDS) pair of patients. All patients were below the age of 60 years at the time of transplantation. Eight patients received myeloablative conditioning with busulfan and cyclophosphamide (BuCy), whereas one AML patient pair received reduced-intensity conditioning with fludarabine, melphalan, and anti-thymocyte globulin.

All patients in the validation set were below the age of 65 years at the time of transplantation. Two-thirds of the patients were diagnosed with acute leukemia, including 38 (42.7%) with AML and 21 (23.6%) with ALL, whereas 20 patients (22.5%) had benign hematologic disorders, including MDS ($n = 7$), primary myelofibrosis ($n = 4$), severe aplastic anemia ($n = 6$), paroxysmal nocturnal hemoglobinuria ($n = 2$), and hemophagocytic lymphohistiocytosis ($n = 1$). The most frequently used conditioning regimen was BuCy ($n = 36$), followed by non-myeloablative fludarabine, busulfan, and anti-thymocyte globulin ($n = 30$). Peripheral blood was the major source of stem cells in both cohorts. All patients received GVHD prophylaxis, per standard protocol (see Supplementary methods).

**Table 1**
Patient characteristics.

| Characteristic | Discovery set ($n = 10$) | Validation set ($n = 89$) |
|---|---|---|
| Median age at alloHSCT, years (range) | 32 (16–57) | 44 (16–64) |
| Sex, *n* (%) | | |
| Male | 3 (30) | 53 (59.6) |
| Female | 7 (70) | 36 (40.4) |
| Diagnosis, *n* (%) | | |
| AML/ALL | 8 (80) | 59 (66.3) |
| CML/MDS/PMF | 2 (20) | 12 (13.5) |
| Other malignant | 0 (0) | 9 (10.1)[a] |
| Other benign | 0 (0) | 9 (10.1)[b] |
| Conditioning, *n* (%) | | |
| Myeloablative[c] | 8 (80) | 37 (41.6) |
| Reduced-intensity[d] | 2 (20) | 20 (22.5) |
| Nonmyeloablative[e] | 0 (0) | 32 (36) |
| Stem cell source, *n* (%) | | |
| BM | 1 (10) | 8 (9) |
| PBSC | 9 (90) | 81 (91) |
| Donor relationship and HLA match, *n* (%) | | |
| Related | | |
| Full match | 2 (20) | 48 (53.9) |
| Haploidentical | 0 (0) | 3 (3.4)[f] |
| Unrelated | | |
| 10/10 match | 6 (60) | 17 (19.1) |
| 9/10 match | 2 (20) | 16 (18) |
| 8/10 match | 0 (0) | 5 (5.6) |
| Donor sex, *n* (%) | | |
| Male | 8 (80) | 63 (70.8) |
| Female | 2 (20) | 26 (29.2) |
| Median donor age, years (range) | 32 (25–46) | 37 (8–77) |
| GVHD prophylaxis, *n* (%) | | |
| CNI | 1 (10) | 11 (12.4) |
| CNI + MTX | 9 (90) | 36 (40.4) |
| CNI + MMF | 0 (0) | 1 (1.1) |
| CNI + ATG | 0 (0) | 22 (24.7) |
| CNI + MTX + ATG | 0 (0) | 19 (21.3) |

alloHSCT, allogeneic hematopoietic stem cell transplantation; AML, acute myeloid leukemia; ALL, acute lymphoblastic leukemia; CML, chronic myeloid leukemia; MDS, myelodysplastic syndrome; PMF, primary myelofibrosis; BM, bone marrow; PBSC, peripheral blood stem cell; GVHD, graft-versus-host disease; CNI, calcineurin inhibitor; MTX, methotrexate; MMF, mycophenolate mofetil; ATG, anti-thymocyte globulin.

[a] Other malignant diseases include non-Hodgkin lymphoma ($n = 7$), aggressive NK cell leukemia ($n = 1$), and T-cell prolymphocytic leukemia ($n = 1$).

[b] Other benign diseases include severe aplastic anemia ($n = 6$), paroxysmal nocturnal hemoglobinuria ($n = 2$), and hemophagocytic lymphohistiocytosis ($n = 1$).

[c] Myeloablative conditioning regimens include BuCy and FluCyTLI.

[d] Reduced-intensity conditioning regimens include FluBuATG (fludarabine 30 mg/m$^2$ × 6 days and busulfan 3.2 mg/kg × 2 days), FluCyATG, and FluMelATG.

[e] Nonmyeloablative conditioning regimen includes FluBuATG (fludarabine 30 mg/m$^2$ × 4 days plus busulfan 0.8 mg/kg × 4 days).

[f] Haploidentical donors include one parent, one sibling, and one child of the respective recipients.

### 3.2. Discovery of candidate aGVHD protein biomarkers

Of 202 unique proteins identified in the proteome profiling of pooled plasma in the discovery phase, the levels of 15 were at least 1.5-fold higher in the GVHD-positive group compared with the GVHD-negative group (Table S1). Of these 15 proteins, 5 were eliminated from consideration because the corresponding proteotypic peptides showed low intensity in MS and MS/MS analyses, thus increasing the likelihood of inaccurate quantification [25]. The remaining 10 candidate proteins corresponded to 34 proteotypic peptides (Table S2). After evaluating the suitability of each peptide for subsequent experiments, 20 of the peptides were selected for LC-MRM MS analysis and quantification of their relative abundance in individual discovery set plasma samples. The resulting data revealed seven proteins exhibiting significantly

**Table 2**
Univariable Cox proportional hazards regression analysis of aGVHD and NRM risk using the validation set.

| Variable | aGVHD risk | | NRM risk | |
|---|---|---|---|---|
| | HR (95% CI) | *P* value | HR (95% CI) | *P* value |
| Age at alloHSCT: ≥45 years vs. < 45 years | 1.58 (0.85–2.96) | 0.15 | 1.18 (0.49–2.86) | 0.709 |
| Sex: female vs. male | 0.96 (0.51–1.8) | 0.889 | 0.78 (0.31–1.97) | 0.605 |
| Diagnosis: malignant vs. benign | 0.61 (0.31–1.21) | 0.156 | 0.56 (0.22–1.41) | 0.217 |
| Stem cell source: PBSC vs. BM | 0.84 (0.3–2.36) | 0.737 | 0.44 (0.13–1.53) | 0.198 |
| Donor relationship: unrelated vs. related | 1.57 (0.84–2.94) | 0.155 | 3.83 (1.47–10) | 0.006 |
| HLA match: mismatched vs. matched | 1.84 (0.96–3.54) | 0.066 | 2.43 (1–5.88) | 0.049 |
| Donor sex: female vs. male | 1.03 (0.52–2.02) | 0.933 | 1.07 (0.41–2.78) | 0.89 |
| Donor age: ≥40 years vs. < 40 years | 0.85 (0.45–1.6) | 0.616 | 0.29 (0.1–0.88) | 0.029 |
| Conditioning: MAC/RIC vs. NMAC | 1 (0.52–1.92) | 0.989 | 0.64 (0.27–1.56) | 0.328 |
| GVHD prophylaxis | | | | |
| CNI + MTX/MMF vs. CNI alone | 0.21 (0.09–0.49) | < 0.001 | 0.22 (0.08–0.67) | 0.007 |
| CNI + ATG ± MTX vs. CNI alone | 0.27 (0.12–0.61) | 0.002 | 0.23 (0.08–0.65) | 0.006 |
| Biomarkers: high vs. low | | | | |
| β2-Microglobulin | 1.49 (0.8–2.78) | 0.213 | 2.79 (1.07–7.26) | 0.036 |
| LRG | 1.78 (0.94–3.37) | 0.075 | 1.37 (0.57–3.32) | 0.482 |
| Fibrillin-like protein | 1.62 (0.87–3.05) | 0.131 | 1.38 (0.57–3.34) | 0.472 |
| Peroxiredoxin-2 | 1.4 (0.75–2.6) | 0.295 | 1.54 (0.63–3.78) | 0.342 |
| TIMP-1 | 2.04 (1.08–3.85) | 0.028 | 2.93 (1.12–7.65) | 0.028 |
| Plastin-2 | 1.57 (0.84–2.94) | 0.156 | 2.49 (0.96–6.48) | 0.062 |
| REG3α | 1.64 (0.88–3.08) | 0.121 | 4.64 (1.55–13.89) | 0.006 |

aGVHD, acute graft-versus-host disease; NRM, non-relapse mortality; HR, hazard ratio; CI, confidence interval; alloHSCT, allogeneic hematopoietic stem cell transplantation; PBSC, peripheral blood stem cell; BM, bone marrow; MAC, myeloablative conditioning; RIC, reduced-intensity conditioning; NMAC, non-myeloablative conditioning; CNI, calcineurin inhibitor; MTX, methotrexate; MMF, mycophenolate mofetil; ATG, anti-thymocyte globulin.

higher expression levels in plasma from the GVHD-positive group compared with the GVHD-negative group. These proteins were subjected to LC-MRM MS analysis for absolute quantification in the validation set (Table S3). All subsequent analyses were conducted using the validation set.

### 3.3. Predictive value of the post-engraftment biomarker level for aGVHD risk

A total of 40 patients (44.9%) in the entire validation set developed aGVHD: overall clinical grade I in 9 (10.1%), grade II in 14 (15.7%), grade III in 12 (13.5%), and grade IV in 5 (5.6%) patients. Plasma samples were obtained a median of 16 (range, 12–29) days after alloHSCT and a median of 4 (range, 0–16) days from the engraftment day (Fig. S2). Univariable analysis indicated that only GVHD prophylaxis and the level of tissue inhibitor of metalloproteinase 1 (TIMP-1) correlated significantly with aGVHD risk (Table 2). Patients with high TIMP-1 levels were approximately twice as likely to experience aGVHD compared to patients with low TIMP-1 levels. All other candidate biomarkers showed a non-significant trend toward increased aGVHD risk in patients with high levels as compared to patients with low levels. After adjustment for the most important clinical characteristics, leucine-rich α-2-glycoprotein (LRG), fibrillin-like protein, and TIMP-1 showed a significant association with aGVHD risk (Table S4). Using a predefined two-step variable selection algorithm, we constructed an optimal multivariable Cox model including two clinical characteristics and two biomarkers. In line with the univariable analysis results, the model indicated that patients with high TIMP-1 and plastin-2 levels had 2.3 and 1.7 times greater risk of developing aGVHD, respectively, compared to patients with low levels, independent of the other covariates (Table S5). No significant correlation between biomarker level and maximal grade of aGVHD was observed among the 40 patients who developed aGVHD (Fig. S3).

### 3.4. Predictive value of the post-engraftment biomarker level for NRM risk

During a median follow-up of 7.1 years, 20 patients (22.5%) died without relapse. Univariable analysis showed correlations between NRM risk and donor-recipient relationship, HLA match, donor age, GVHD prophylaxis, and plasma levels of β2-microglobulin, TIMP-1, and regenerating islet-derived protein 3-α (REG3α) (Table 2). Among the biomarkers identified, TIMP-1 and REG3α were predictive of NRM risk even after adjustment for clinical variables (Table S6). Similar to the case of aGVHD risk analysis, all other biomarkers exhibited a trend toward increased NRM risk in patients with high levels as compared to patients with low levels. The optimal multivariable model included five variables (two clinical predictors and three biomarkers), three of which were also included in the optimal model to predict aGVHD risk (Table S7). High plasma level of each of the three biomarkers included in the model was associated with a > 2-fold higher risk of NRM compared to patients with low plasma level of that biomarker; however, the difference was significant only for REG3α.

### 3.5. Association of biomarker panel score with aGVHD and NRM risk

Using the biomarkers included in at least one of the two optimal Cox models (one each for aGVHD and NRM risk; i.e., TIMP-1, plastin-2, and REG3α), we defined a new composite variable, the biomarker panel score, as the number of markers present at high levels in a given patient. Therefore, the biomarker panel score ranged from 0 to 3. It was necessary to reduce the whole set of seven candidate biomarkers into a core subset-based score because many of the candidate markers showed high pairwise correlations in terms of plasma level (Fig. 1). Notably, the biomarker panel score correlated significantly with both the risk of aGVHD and NRM, both before and after adjustment for clinical characteristics (Table 3).

Nested-model likelihood ratio testing indicated the biomarker panel score contributed significantly to model performance when it was added to the model including clinical characteristics only for both aGVHD and NRM risk ($P = 0.023$ and $< 0.001$, respectively). The distribution of 5-CVC indices generated using 200 independent random splittings of the dataset also indicated that the discrimination capability of the model including both clinical characteristics and biomarker panel score was significantly greater than that of the model including only clinical characteristics (Fig. 2). Addition of the biomarker panel score resulted in correct reclassification of 29.6% of patients who developed aGVHD within 6 months of alloHSCT and 8.8% of patients who did not develop aGVHD within this timeframe; the NRI index was 38.3%,

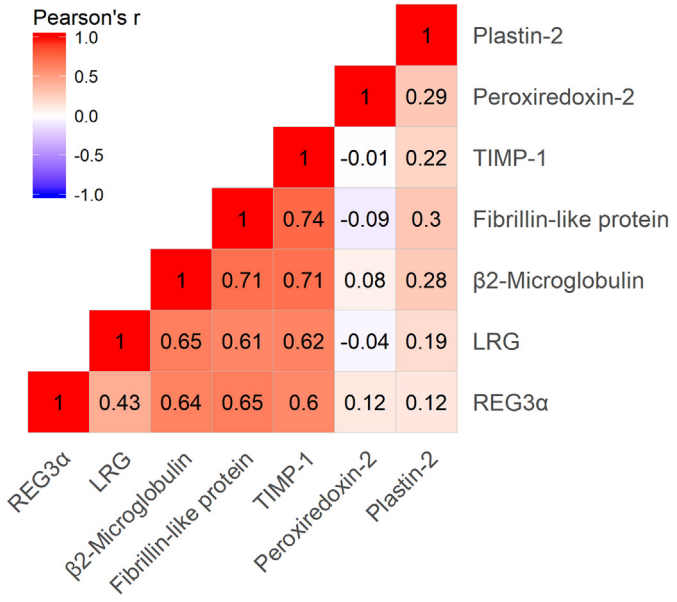


**Fig. 1.** Pairwise correlation of candidate protein biomarker plasma level in the validation set ($n$ = 89). For the full name of each protein, refer to Table S1.

**Table 3**
Association of biomarker panel score with cause-specific risk of aGVHD and NRM.

| Model | aGVHD risk | | NRM risk | |
|---|---|---|---|---|
| | HR (95% CI) | *P* value | HR (95% CI) | *P* value |
| Univariable | 1.57 (1.13–2.18) | 0.008 | 2.68 (1.57–4.59) | < 0.001 |
| Multivariable[a] | 1.55 (1.07–2.25) | 0.022 | 2.76 (1.52–4.99) | 0.001 |

aGVHD, acute graft-versus-host disease; NRM, non-relapse mortality; HR, hazard ratio; CI, confidence interval.

[a] The presented HR is adjusted for donor-recipient HLA allele match and GVHD prophylaxis regimen in the model for aGVHD risk and for donor-recipient relationship and GVHD prophylaxis regimen in the model for NRM risk, as per the predefined variable selection algorithm.

although this was not significant ($P$ = 0.074; Table S8). By contrast, the model including both clinical characteristics and biomarker panel score correctly reclassified 32.7% of patients who died without relapse and 21% of patients who did not die without relapse within 1 year after alloHSCT as compared to the model including clinical characteristics only. The degree of reclassification was significant, with a NRI index of 53.7% ($P$ = 0.032; Table S9).

### 3.6. Cumulative incidence of aGVHD and NRM based on biomarker levels

As shown in Figs. S4 and S5, patients with high plasma biomarker levels consistently tended toward a higher cumulative incidence of both aGVHD and NRM compared to patients with low plasma levels of all of the candidate biomarkers. In particular, levels of TIMP-1, plastin-2, and REG3α, which together comprised the biomarker panel score, correlated significantly with cumulative NRM incidence (Fig. S5), but the level of no single biomarker correlated significantly with the cumulative incidence of aGVHD. Likewise, patients with a high biomarker panel score exhibited significantly higher cumulative NRM incidence than patients with a low biomarker panel score (Fig. 3). The difference in cumulative incidence of aGVHD based on biomarker panel score was not significant, although there was an overall trend toward a higher incidence in patients with higher scores. In line with these results, the Fine-Gray model also revealed significant NRM subdistribution hazard ratios (HRs) for the biomarker panel score in both univariable and multivariable analyses (Table 4).

Because the biomarker panel score showed a significant correlation with cause-specific hazard of developing aGVHD (Table 3) but not with the subdistribution HR of developing aGVHD (Table 4), we evaluated whether this discrepancy was caused by an increased risk competing with aGVHD (i.e., death without aGVHD) in patients with a high biomarker panel score. Indeed, the subdistribution HR (95% CI) for death without aGVHD was 1.49 (0.87–2.53) in the univariable model and 1.61 (0.95–2.73) in the multivariable model, both of which were numerically higher than the subdistribution HRs for aGVHD. Our data suggest that the significant cause-specific HR for aGVHD of the biomarker panel score did not translate into a significantly higher actual incidence of aGVHD in higher-score patients because the score also correlated with the incidence of death unrelated to aGVHD, an event competing with aGVHD. Among the 21 patients who died without aGVHD, the most common cause of death was relapse ($n$ = 10), followed by infection ($n$ = 4) and multiple organ dysfunction syndrome ($n$ = 3).

### 3.7. Organ-specific association between biomarker level and aGVHD risk

Certain plasma proteins may reflect injury to specific target organs and therefore could be used as diagnostic biomarkers for organ-specific aGVHD [8,9]. Based on this concept, we hypothesized that predictive biomarkers might also indicate organ-specific aGVHD risk. Thus, we separately compared the cumulative incidence of aGVHD involving each of the three major target organs (i.e., skin, gastrointestinal tract, and liver) between patients with high or low levels of each of seven

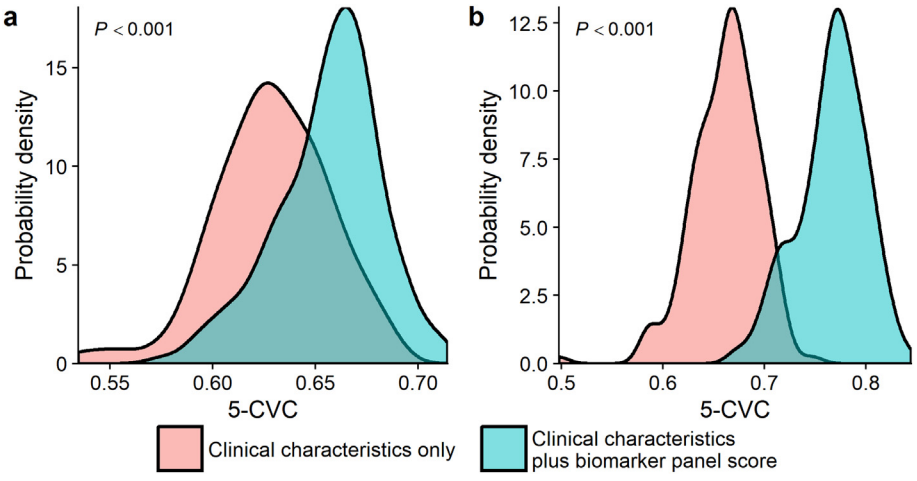


**Fig. 2.** Distribution of the 5-CVC indices generated 200 times by random splitting of the validation set into five subsets for the Cox model containing clinical characteristics only and the Cox model containing clinical characteristics plus biomarker panel score to predict the risk of aGVHD (a) and NRM (b). Note that the area under each curve is equal to 1. 5-CVC, 5-fold cross-validated C; aGVHD, acute graft-versus-host disease; NRM, non-relapse mortality.

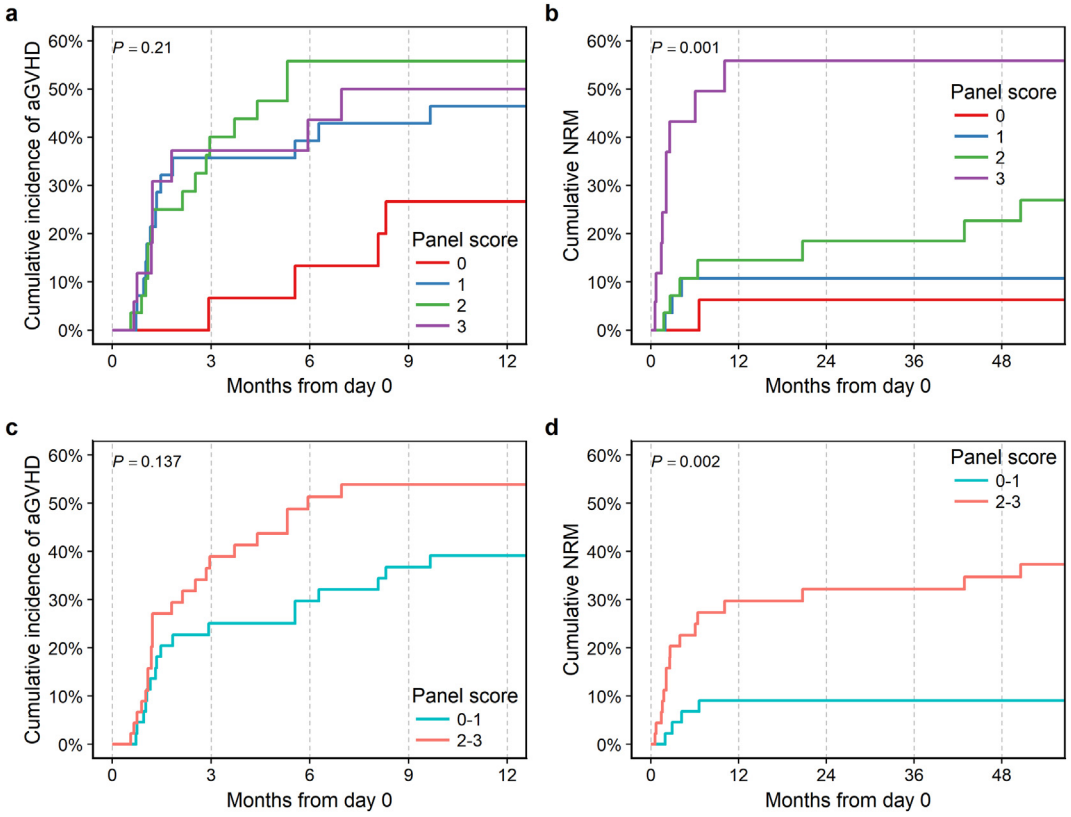


**Fig. 3.** Cumulative incidence of aGVHD (a and c) and NRM (b and d) according to biomarker panel score. aGVHD, acute graft-versus-host disease; NRM, non-relapse mortality.

**Table 4**
Association of biomarker panel score with sub-distribution risk of aGVHD and NRM.

| Model | aGVHD risk | | NRM risk | |
|---|---|---|---|---|
| | HR (95% CI) | *P* value | HR (95% CI) | *P* value |
| Univariable | 1.3 (0.98–1.71) | 0.068 | 2.68 (1.52–4.74) | 0.001 |
| Multivariable[a] | 1.2 (0.85–1.69) | 0.29 | 2.56 (1.36–4.81) | 0.003 |

aGVHD, acute graft-versus-host disease; NRM, non-relapse mortality; HR, hazard ratio; CI, confidence interval.

[a] The presented HR is adjusted for donor-recipient HLA allele match and GVHD prophylaxis regimen in the model for aGVHD risk and for donor-recipient relationship and GVHD prophylaxis regimen in the model for NRM risk, as per the predefined variable selection algorithm.

candidate biomarkers. No significant organ-specific correlation between levels of the respective biomarkers and aGVHD risk was observed, although visual inspection of the cumulative incidence curves suggested a potential predictive value of LRG for risk of skin aGVHD, REG3α for gastrointestinal aGVHD, and TIMP-1 for both skin and liver aGVHD (Supplementary Figs. S6–S8). Likewise, the biomarker panel score did not significantly correlate with the risk of organ-specific aGVHD, but the risk of skin and liver aGVHD tended to be higher in patients with high biomarker panel scores (Fig. S9).

## 4. Discussion

In the discovery set of the present study, we used an LC-MS/MS-based quantitative proteomics method to comprehensively profile the plasma proteomes of aGVHD and control patients who underwent alloHSCT. A total of seven candidate biomarkers were identified, and their predictive value was validated in terms of aGVHD and NRM risk using post-engraftment plasma samples from an independent patient cohort. Three of the candidate biomarkers (TIMP-1, plastin-2, and REG3α) were used to develop a simple combined biomarker panel score, which accurately and precisely stratified patients according to aGVHD and NRM risk post-alloHSCT. We also demonstrated that use of the biomarker panel score in combination with established clinical predictors (e.g., donor-recipient HLA allele match, donor-recipient relationship, and GVHD prophylaxis regimen) significantly improved the discriminatory performance of the Cox model in terms of predicting aGVHD and NRM risk. As aGVHD represents a leading cause of NRM in patients undergoing alloHSCT, it is clinically plausible that the predictive value of the aGVHD risk biomarkers translates directly into predictive value for NRM risk.

When analyzed individually, only some of the selected biomarkers identified in our study reached statistical significance in terms of predictive value for aGVHD or NRM risk, but the biomarker panel score correlated significantly with both aGVHD and NRM risk. Given the heterogeneous and complex nature of aGVHD pathophysiology, it is unlikely that a single protein would reflect the overall pathogenesis of aGVHD [26]. Instead, combining multiple biomarkers into a panel-based scoring system appears to be a more promising strategy. Indeed, a series of previous studies demonstrated that combining multiple biomarkers in a panel-based prediction model enabled both discrimination of patients with/without aGVHD and prediction of the risk of unresponsiveness to corticosteroid therapy and subsequent NRM in patients who developed aGVHD following alloHSCT [6,10,12]. In the present study, we extended the clinical utility of the plasma-based biomarker panel by demonstrating that it can also be used to predict aGVHD and NRM risk even before the onset of clinical manifestations.

It should be noted that the HR for NRM was higher than that for aGVHD in both the Cox and Fine-Gray model analyses, which suggests an additional role for the biomarker panel score in predicting NRM not associated with aGVHD. In line with this reasoning, the Fine-Gray

model for death in patients without aGVHD indicated that the biomarker panel score correlated more strongly with the incidence of death unassociated with aGVHD than with the incidence of aGVHD itself. Therefore, it is likely that the predictive value of the biomarker panel score in terms of the cumulative incidence of aGVHD was negatively affected by its stronger predictive value for the risk of a competing event (i.e., death without aGVHD), resulting in an attenuated subdistribution HR for aGVHD in the Fine-Gray model. This is a commonly encountered situation in competing risks studies [22]; however, we cannot exclude the possibility that our competing risks analysis was not sufficiently empowered to detect the relationship between the biomarker panel score and the cumulative incidence of aGVHD due to the relatively small number of patients in the validation set.

Of the three biomarkers for which the levels were included in calculating the panel score, REG3α is a specific marker of gastrointestinal aGVHD [7,9]. REG3α, a C-type lectin secreted by Paneth cells, mediates antibacterial mechanisms in the intestinal crypt microenvironment downstream of interleukin-22 signaling [9,27]. Since its discovery, several studies have adopted REG3α as a component of a systemic biomarker panel to predict nonresponse to therapy and mortality in newly diagnosed aGVHD patients, with promising results [10,12]. Consistent with these results, visual comparison of the cumulative incidence curves for gastrointestinal aGVHD between patients with high and low plasma REG3α levels in the present study indicated this protein has a high predictive value for intestinal aGVHD. Given that our study was not statistically powered to draw reliable target organ-specific conclusions, the predictive value of plasma REG3α for intestinal aGVHD should be confirmed in future studies.

TIMP-1 belongs to a family of four endogenous metalloproteinase inhibitors (TIMP-1 through TIMP-4) that exhibit a broad range of biological activities [28]. Evidence accumulated over the last few decades has shed light on the fundamental roles of matrix metalloproteinases and their endogenous inhibitors, including TIMP-1, in immune regulation [29]. Accordingly, dysregulation in metalloproteinase activity has been linked to various inflammatory and autoimmune disorders, such as inflammatory bowel disease, granulomatous skin disorder, and aGVHD [29,30]. Furthermore, in previous studies, a synthetic metalloproteinase inhibitor significantly ameliorated the phenotypic and pathologic severity of aGVHD as well as mortality in animal models [31,32]. Therefore, although the predictive or prognostic role of TIMP-1 for aGVHD has not been evaluated in patients undergoing alloHSCT prior to this study, the theoretical basis and evidence provided by this research makes it a plausible biomarker for aGVHD.

The third protein comprising the biomarker panel score, plastin-2, is an actin-binding phosphoprotein that mediates transport of the T-cell activation molecules CD25 and CD69 to the cell surface in response to co-stimulation via T-cell receptor/CD3 plus CD2 or CD28 [33]. Plastin-2 governs bundling of the F-actin cytoskeleton, which is crucial for immune synapse formation, polarization, and migration in chemokine-stimulated T cells [34,35]. Importantly, co-stimulation-induced plastin-2 phosphorylation is inhibited by the glucocorticoid dexamethasone, which consequently prevents maturation of immune synapses [36]. Therefore, the glucocorticoid's anti-aGVHD mechanism can be explained at least in part by its inhibitory effect on plastin-2-mediated immune synapse formation. Considered collectively, these observations highlight the key role of plastin-2 in activating T cells that mediate the pathogenesis of aGVHD. Nevertheless, how plastin-2 is released into the plasma during the early post-alloHSCT period and how its plasma level is related to alloimmune activity remain to be determined.

This study has several limitations. First and most important, we could not externally validate the predictive role of the biomarker panel score due to the lack of an additional independent cohort. Although we employed an alternative strategy with internal validation methods, such as 5-fold cross-validation and bootstrapping, this was not sufficient for validating a new prediction model. Additionally, the small size of our validation set did not allow us to elucidate target organ-specific associations of each biomarker. For the same reason, we could not assess whether the identified biomarkers are specific for aGVHD or also indicative of other alloHSCT-related complications, such as infection or veno-occlusive disease. The lack of a relationship between candidate biomarker levels and clinical grade of aGVHD also limits the value of the identified candidate biomarkers. Lastly, although the way the biomarker panel score was defined in this study is intuitive and easy to implement, the optimal thresholds for classifying patients into high- and low-level groups should be determined using a larger number of cases.

Despite the above-mentioned limitations, to our knowledge, this is the first study to employ an LC-MS/MS-based quantitative proteome profiling method, which is currently state-of-the-art in proteomics research, to develop a predictive plasma-based biomarker panel for aGVHD using samples obtained at the preclinical stage. The results of our study indicated a consistent tendency toward higher aGVHD and NRM risk in patients with high plasma levels of seven candidate biomarkers. The three-protein combined biomarker panel enabled prediction of aGVHD and NRM risk independent of clinical characteristics and significantly improved the model performance. Another important clinical implication of GVHD-specific biomarkers is that they can be therapeutically targeted, as exemplified by metalloproteinase inhibitor and tumor necrosis factor blockade [31,32,37]. Continuing efforts to identify a comprehensive catalog of aGVHD biomarkers could therefore pave a new path in the search for novel prophylactic and therapeutic agents.

## Conflict of interest

The authors declare that they have no conflict of interest.

## Authors' contributions

JS, KD, JWK, SHS, and IK conceptualized and designed the study. SSY, SP, and IK provided patient information and materials. KD and JWK selected patients and samples used in the discovery phase. JS and KD selected patients and samples used in the validation phase. JS, KD, and JWK reviewed medical records to retrieve clinical data. KD performed the laboratory work. JS performed statistical analyses. JS and KD wrote the manuscript. DH, JWK, KKK, SHS, and IK provided critical input on data analysis. All authors contributed to the interpretation of results. SHS and IK approved the final version of the manuscript.

## Acknowledgments

This work was supported by grant no. 0320150150 from the Seoul National University Hospital Research Fund and in part by grant no. 0420160240 from the Seoul National University Hospital Research Fund. This manuscript was edited by a native English-speaking expert associated with BioScience Writers LLC, Houston, TX, USA.

## Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.bcmd.2018.10.001.

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