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Biomarkers

Association of Plasma CD163 Concentration with De Novo–Onset Chronic Graft-versus-Host Disease

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A B S T R A C T

Chronic graft-versus-host disease (GVHD) is the leading cause of long-term morbidity and mortality after allogeneic hematopoietic cell transplantation. To identify prognostic plasma proteins associated with de novo– or quiescent-onset chronic GVHD (cGVHD), we performed a discovery and validation proteomic study. The total study cohort included 167 consecutive patients who had no clinical evidence of GVHD under minimum glucocorticoid administration and had available plasma samples obtained at 80 ± 14 days after transplantation. We first used high-throughput mass spectrometry to screen pooled plasma using 20 cases with subsequent cGVHD and 20 controls without it, and we identified 20 candidate proteins. We then measured 12 of the 20 candidate proteins by ELISA on the same individual samples and identified 4 proteins for further verification (LGALS3BP, CD5L, CD163, and TXN for de novo onset, and LGALS3BP and CD5L for quiescent onset). The verification cohort included 127 remaining patients. The cumulative incidence of de novo–onset cGVHD was higher in patients with higher plasma soluble CD163 concentrations at day 80 than those with lower concentrations (75% versus 40%, $P = .018$). The cumulative incidence of de novo– or quiescent-onset cGVHD did not differ statistically according to concentrations of the 3 other proteins at day 80. CD163 is a macrophage scavenger receptor and is elevated in oxidative conditions. These results suggest that monocyte or macrophage activation or increased oxidative stress may contribute to the pathogenesis of cGVHD.

INTRODUCTION

Chronic graft-versus-host disease (cGVHD) occurs in approximately 30% to 50% of patients after allogeneic hematopoietic cell transplantation (HCT) and is the leading cause of late morbidity and mortality [1]. The disease usually occurs beyond 80 days after HCT and the median onset is 5 months after HCT [2]. This complication is thought to occur because the donor immune system recognizes recipient tissues, causing inflammation and fibrosis [3,4].

High-throughput mass spectrometry is a powerful, comprehensive, and reduced-bias approach to identify proteomic profiles in many diseases [5]. This approach has been very successful in identifying plasma biomarker proteins that correlate with activity and treatment response of acute graft-versus-host disease (GVHD) [6-11] and diagnosis and severity of cGVHD [12,13]. To date, prognostic plasma biomarker proteins associated with subsequent onset of cGVHD have not been examined by mass spectrometry. Furthermore, few studies have considered the influence of glucocorticoid dosing when the sample was obtained, despite the large influence of glucocorticoids on gene expression and the concentrations of plasma proteins [12,14]. We hypothesized that comparing plasma proteomic profiles before the onset of cGVHD between patients with and without subsequent cGVHD could identify prognostic proteins associated with the development of cGVHD.

METHODS

Study Design

To identify prognostic plasma proteins associated with the subsequent de novo or quiescent-onset cGVHD, we performed a discovery and validation

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proteomic study using high-throughput mass spectrometry. The study was carried out in 3 phases: (1) an initial discovery phase testing pooled plasma samples by mass spectrometry, (2) confirmation of the candidate proteins in individual samples of the discovery cohort by using ELISA, and (3) verification of candidate proteins in the remaining patients.

Patients and Sample Collection

Patients gave written consent allowing blood sample collection and the use of medical records for research in accordance with the Declaration of Helsinki. The institutional review board of the Fred Hutchinson Cancer Research Center approved the study. Patients were recruited before transplantation, and blood samples were prospectively collected and cryopreserved at 80 ± 14 days after transplantation. Plasma was collected in EDTA and aliquoted in .5-mL tubes and stored at –80°C within 2 hours of phlebotomy. Acute GVHD was diagnosed and graded according to the previously described criteria [15,16]. Chronic GVHD was diagnosed by the National Institutes of Health (NIH) consensus criteria [17].

The total study cohort included 167 consecutive relapse-free patients who met all of the following criteria: (1) allogeneic HCT at the Fred Hutchinson Cancer Research Center/Seattle Cancer Care Alliance between April 2003 and December 2011, (2) available plasma samples at day 80 ± 14 days after HCT, (3) no prior cGVHD at the time of sample collection, (4) no active GVHD at the time of sample collection, and (5) prednisone-equivalent steroid doses ≤5 mg daily at sample collection. Patients were eligible regardless of the indication for HCT, conditioning regimen, graft source, donor relationship, and HLA-matching between the donor and recipient.

Discovery and Verification Cohorts

Patients who subsequently developed systemically treated cGVHD were declared cases, whereas patients without subsequent cGVHD were declared controls. The discovery cohort included 2 pools from 40 patients selected from the 167 patients described above: 1 pool of 20 patients with prior acute GVHD and another pool of 20 without prior acute GVHD. Two independent intact protein analyses (IPA) were done with pooled plasma samples according to presence or absence of prior acute GVHD. Among 20 patients with no prior acute GVHD (IPA1), 10 patients were cases and 10 were controls. Likewise, among 20 patients with prior acute GVHD (IPA2), 10 were cases and 10 were controls. Cases and controls were matched for recipient gender (Table 1). The verification cohort included the 127 remaining patients.

Mass Spectrometric Analysis

Samples were depleted of the 6 most abundant proteins (albumin, IgG, IgA, transferrin, haptoglobin, and antitrypsin), pooled, and labeled with light acrylamide (cases: subsequent cGVHD-positive) or with a heavy 1,2,3-^{13}C-acrylamide (controls: subsequent cGVHD-negative) [5,18]. The pool of cases and the pool of controls were mixed together before further processing and IPA analysis. Proteins were separated by an automated online 2D-HPLC system controlled by Workstation Class-VP 7.4 (Shimadzu Corporation, Kyoto, Japan) [5,18]. Separation consisted of anion exchange chromatography followed by reverse-phase chromatography. In-solution tryptic digestion was conducted with lyophilized aliquots from the reverse-phase (second-dimension) fractionation step [13,19]. Aliquots were subjected to tandem mass spectrometry shotgun analysis using an LTQ-Orbitrap (Thermo, Waltham, MA) mass spectrometer coupled with a NanoLC-1D (Eksigent, Dublin, CA) on a 2-hour gradient [13,19].

Data Processing

Raw machine output files from all mass spectrometry runs were converted to mzXML files and searched with X!Tandem [20] configured with the k-score scoring algorithm [21] against the Uniprot database. Peptide identifications were assigned probability by PeptideProphet [22], with a model built on all sample fractions together. As a conservative quality filter, only those identifications with an individual identification probability of .95 or higher were retained. The Q3 algorithm for labeled quantitation [23] was applied to estimate quantitative ratios for all cysteine-containing peptides, with a correction for the overlap between light and heavy isotopic peaks. The Qurate algorithm [19] within the msInspect platform [24] was applied in an automated fashion to locate and remove peptide quantitative events likely to be incorrect because of apparent coeluting peptides, poor isotopic peak distribution, or missing isotopic peaks.

The remaining peptide identifications were provided to ProteinProphet [25] for protein inference. Because only high-quality peptide identifications were used, protein probability assignments were ignored, except to

Table 1
Patient Characteristics

Characteristic	Discovery Cohort (n = 40)				Verification Cohort (n = 127)	
	IPA 1: Prior Acute GVHD –		IPA 2: Prior Acute GVHD +		Subsequent cGVHD	
	Subsequent cGVHD		Subsequent cGVHD			
	Yes (n = 10)	No (n = 10)	Yes (n = 10)	No (n = 10)	Yes (n = 82)	No (n = 45)
Prior acute GVHD	0 (0)	0 (0)	10 (100)	10 (100)	41 (50)	24 (53)
Patient age at transplantation, median (range), yr	54 (36-63)	43 (32-59)	48 (28-71)	48 (19-64)	55 (26-73)	52 (20-69)
Median months from sample collection to cGVHD (range)	2.7 (1.4-5.0)	NA	5.2 (.5-16)	NA	4.9 (.5-31)	NA
Patient gender						
Male	5 (50)	5 (50)	5 (50)	5 (50)	58 (71)	25 (56)
Female	5 (50)	5 (50)	5 (50)	5 (50)	24 (29)	20 (44)
Disease risk at transplantation*						
Standard	5 (50)	5 (50)	3 (30)	5 (50)	27 (33)	17 (38)
High	5 (50)	5 (50)	7 (70)	5 (50)	55 (67)	28 (62)
HLA and donor type						
Matched related	7 (70)	5 (50)	6 (60)	5 (50)	33 (40)	19 (42)
Matched unrelated	2 (20)	3 (30)	4 (40)	3 (30)	36 (44)	17 (38)
HLA mismatched	1 (10)	2 (20)	0 (0)	2 (20)	13 (16)	9 (20)
Conditioning regimen						
High intensity	4 (40)	7 (70)	5 (50)	8 (80)	45 (55)	21 (47)
Reduced intensity	6 (60)	3 (30)	5 (50)	2 (20)	37 (45)	24 (53)
Graft source						
Bone marrow	0 (0)	2 (20)	0 (0)	4 (40)	6 (7)	11 (24)
Mobilized blood cells	10 (100)	8 (80)	10 (100)	6 (60)	76 (93)	32 (71)
Umbilical cord blood	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	2 (4)
T cell depletion						
Antithymocyte globulin	0 (0)	1 (10)	0 (0)	2 (20)	1 (1)	1 (2)
Alemtuzumab	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)
Immunosuppressive drugs at sample collection						
Calcineurin inhibitor	10 (100)	10 (100)	8 (80)	10 (100)	80 (98)	41 (91)
Mycophenolate mofetil	1 (10)	0 (0)	2 (20)	0 (0)	21 (26)	12 (27)
Sirolimus	0 (0)	0 (0)	0 (0)	0 (0)	3 (4)	1 (2)

Data presented are n (%) unless otherwise indicated.
NA indicates not available.
* The standard-risk category included chronic myeloid leukemia in chronic phase, acute leukemia in first remission, myelodysplastic syndrome without excess blasts, and nonmalignant diseases. The high-risk category included all other diseases and stages.