

Accurate diagnosis of acute graft-versus-host disease using serum proteomic pattern analysis

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Objective. The rapid diagnosis of acute graft-versus-host disease (GVHD) following allogeneic hematopoietic cell transplantation (HCT) is important for optimizing the management of this life-threatening complication. Current diagnostic techniques are time-consuming and require invasive tissue sampling. We investigated serum protein pattern analysis using surface-enhanced laser desorption ionization time-of-flight (SELDI-TOF) mass spectrometry as a tool to diagnose GVHD.

Patients and Methods. Eighty-eight serum samples were obtained from 34 patients undergoing HCT either pretransplant (n = 28 samples) or at various time points posttransplant (n = 60 samples), including 22 samples obtained on the day of onset of acute GVHD symptoms. Serum proteomic spectra generated from a “training set” of known samples were used to identify distinct proteomic patterns that best categorized a sample as either pretransplant, posttransplant non-GVHD, or GVHD; these distinct proteomic signatures were subsequently used to classify samples from a masked “test” sample set into the appropriate diagnostic category.

Results. Proteomic pattern analysis accurately distinguished GVHD samples from both posttransplant non-GVHD samples and pretransplant samples (100% specificity and 100% sensitivity in both cases). Furthermore, distinct serum proteomic signatures were identified that distinguished pretransplant from posttransplant non-GVHD samples (100% specificity and 94% sensitivity).

Conclusion. These preliminary data suggest a potential application of SELDI-TOF-based proteomic analysis as a rapid and accurate method to diagnose acute GVHD. © 2006 International Society for Experimental Hematology. Published by Elsevier Inc.

Acute graft-versus-host disease (GVHD) occurs commonly after allogeneic hematopoietic cell transplantation (HCT), affecting 30 to 80% of patients undergoing the procedure [1]. A prompt diagnosis followed by the timely initiation of appropriate immunosuppressive therapy is vital for the reduction of morbidity and mortality associated with this complication. For decades, histopathologic evaluation of

tissue obtained from an involved organ has been the “gold standard” for the diagnosis of acute GVHD. Biopsy procedures to procure tissue are invasive and incur delays of 24 to 48 hours before a diagnosis can be rendered. Furthermore, even when appropriate tissue is available, an unequivocal diagnosis of GVHD using currently available technology is sometimes impossible. Establishing the diagnosis solely on clinical grounds can be challenging, as a number of posttransplant conditions may mimic this complication [2]. Therefore, the development of a noninvasive method to quickly and accurately diagnose acute GVHD could facilitate the management of this complication.

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The development of serum proteomic analysis as a diagnostic tool is based on the premise that systemic as well as organ-specific alterations related to pathologic states are accompanied by quantitative and qualitative alterations in serum protein and polypeptide profiles. These alterations in the serum “proteome” serve as surrogate biomarkers that can be assayed with relative ease and utilized to predict the presence or absence of disease. Advances in the field of mass spectrometry, coupled with the development of powerful bioinformatics tools, have recently made high-throughput protein-based analysis of biologic specimens practical [3]. Proteomic analysis using surface-enhanced laser desorption and ionization time-of-flight (SELDI-TOF) mass spectrometry is emerging as a valuable tool in the field of cancer diagnostics. SELDI-TOF involves the application of a small amount of biological sample (e.g., 1 to 5 mL of serum) to a “protein chip” followed by the addition of an acidic “matrix” that facilitates ionization of immobilized proteins/peptides upon exposure to a laser source. The gaseous ions thus generated are transported through a time-of-flight mass spectrometer, and a unique mass/charge (M/Z) ratio determined for each ion based on its time of flight through the spectrometer. The approximately 15,200 data points generated from each sample are then analyzed to help identify patterns that characterize the presence or absence of a given disease state. A recent study demonstrated that serum proteomic spectra generated by SELDI-TOF mass spectrometry could accurately distinguish nonmalignant ovarian pathology from ovarian neoplasms [4]. The successful application of SELDI-TOF mass spectrometry to the diagnosis of ovarian cancer has led to studies investigating this powerful technology in the noninvasive diagnosis of other malignancies including prostate, breast, and hepatocellular cancer [5–8]. We sought to investigate the utility of SELDI-TOF-based serum proteomic analysis in the diagnosis of GVHD.

Materials and methods

Study population

This study was performed on banked serum samples obtained from patients with solid tumors, hematologic malignancies, and nonmalignant hematologic diseases (e.g., severe aplastic anemia, paroxysmal nocturnal hemoglobinuria) undergoing HCT at the National Heart, Lung and Blood Institute on various Institutional Review Board–approved allogeneic hematopoietic cell transplant protocols.

GVHD and serum samples

Eighty-eight serum samples were obtained from 34 patients undergoing HCT. Twenty-two serum samples were taken from patients on the day acute GVHD developed (between days +21 and +204 posttransplant). Serum samples were obtained at the onset of acute GVHD symptoms, prior to initiation of systemic corticosteroid therapy. Acute GVHD was diagnosed based on classic clinical findings. Histopathologic evaluation of colonic tissue was

obtained and a diagnosis of acute GVHD confirmed when clinically warranted, including in all 12 patients presenting with symptoms of gastrointestinal GVHD (Fig. 1); confounding diagnoses such as cytomegalovirus (CMV) and *C. difficile* colitis were ruled out. The study excluded patients whose symptoms could be partly or wholly explained by another diagnosis (e.g., infectious colitis, drug rash, etc.). GVHD was graded in accordance with standard criteria [9]. Thirty-eight serum samples were obtained after transplantation from asymptomatic patients (i.e., when no symptoms to suggest GVHD existed) to serve as posttransplant controls (between days +5 and +259 posttransplant); 16 of these samples were obtained from patients who subsequently developed GVHD and had serum collected for analysis in the GVHD cohort. Twenty-eight pretransplant serum samples were also obtained for additional comparisons from the same pool of patients that contributed posttransplant samples (GVHD and non-GVHD). Whole blood was collected in “red top” vacutainer tubes with clot activator (BD Diagnostics, Franklin Lakes, NJ, USA); samples were allowed to clot at room temperature and serum collected after centrifugation at 3000 rpm for 10 minutes. All samples were divided into 20- μ L aliquots, stored at -80 C, and thawed immediately prior to analysis.

Proteomic analysis

Proteomic analyses were performed using ProteinChip Weak cation exchange interaction chips (WCX2, Ciphergen Biosystems, Fremont, CA, USA). WCX2 protein arrays were serially pretreated with 10 mM hydrochloric acid and 10 mM ammonium acetate with 0.1% Triton X, then vacuum dried to remove excess fluid. Five mL of undiluted serum was applied to each spot on the protein chip and incubated for 20 minutes. Each protein chip was then washed twice with 200 mL of phosphate-buffered saline and 200 mL of deionized water, the liquid removed, and the protein arrays dried. One mL of energy-absorbing matrix consisting of 3 mg/mL cinnamic acid (Fluka, St. Louis MO, USA) in 50% (v/v) acetonitrile and 0.5% trifluoroacetic acid was then applied

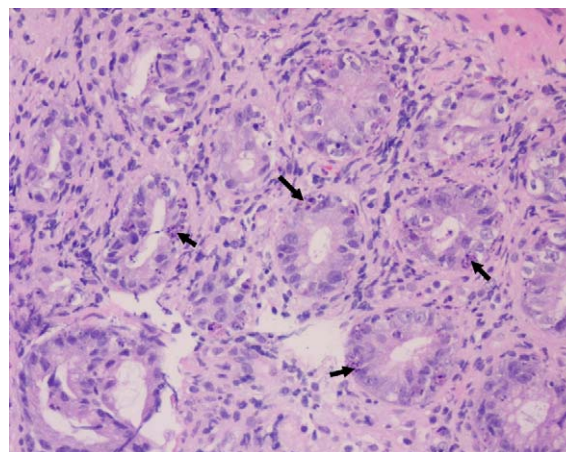


Figure 1. Representative histopathologic findings in acute GI GVHD. Classical histopathologic findings of gastrointestinal GVHD in a patient whose serum protein patterns analyzed by SELDI-TOF mass spectrometry were consistent with acute GVHD. The presence of apoptotic crypt cells accompanied by a relatively sparse mononuclear inflammatory infiltrate was used to make the diagnosis of GVHD. CMV was excluded by immunohistochemical staining in all cases (not shown).

to the protein chip and the chip was dried. The protein arrays were then analyzed on a SELDI-TOF mass spectrometer (CIPHERGEN PBSIIC, Ciphergen Biosystems). GVHD samples and pretransplant and posttransplant controls (no GVHD) were run concurrently, intermingled on the same chip; operators were completely blinded to sample identity.

Analytical procedure

Proteomic spectra generated by SELDI-TOF were analyzed as previously described [4,8] using Proteome Quest beta version 1.0 (Correlogic Systems Inc, Bethesda, MD, USA), a data-mining tool based on genetic algorithms and cluster analysis. A “training set” consisting of “known” samples was used to generate a proteomic signature that best segregated GVHD samples from pretransplant or posttransplant non-GVHD samples. Each proteomic signature consists of a subset of defined mass/charge (M/Z) values whose amplitudes best segregate the groups within the training set. Proteomic signatures identified from the training set were then used to evaluate GVHD and non-GVHD serum in an independent “test set” of masked samples (Fig. 2).

A detailed description of the quality control measures and statistical analysis techniques used has been previously published [4,8,10]. Briefly, the quality of the spectra was assessed by plotting the total ion current of each spectrum and examining the mean and standard deviation of the values. Using stored procedures developed in-house, any spectrum that failed statistical checks was eliminated from modeling and analysis. The pattern discovery module in the Correlogic ProteomeQuest software was used for model building. This utilizes normalized mass spectral data and combines elements from genetic algorithms and a self-organizing adaptive pattern-recognition system to define sample groups into clusters in N-dimensional space through a survival-of-the-fittest

approach. These clusters are plots of Euclidean distance vectors composed of the combined normalized intensities of the randomly sampled M/Z values of the different classification groups. Using the samples held for testing, the distance vectors are calculated and plotted for each sample using only the M/Z values that make up the diagnostic model identified in training. N-dimensional plotting yields the classifications by a determination of which cluster (if any) the sample falls within. This allows classification into a cluster or “unknown” if the sample does not fall within a cluster.

Results

Baseline characteristics of patients from whom the 22 GVHD, 28 pretransplant, and 38 posttransplant non-GVHD serum samples were obtained are listed in Table 1. The groups were well matched with regard to age, sex, and the pretransplant conditioning regimen used. Table 2 lists the location and severity of GVHD in patients who contributed serum samples. All 28 pretransplant samples were obtained from patients who had at least one posttransplant (GVHD or non-GVHD) sample included in the analysis.

Proteomic spectra could be used to identify serum samples as either pretransplant or posttransplant (non-GVHD). A total of 66 samples were used for this analysis (including 48 paired pre- and posttransplant samples from 24 patients). A discriminatory spectral pattern generated from 37 “training samples” correctly classified 28/29 validation or test samples as being either pre- or posttransplant serum (100% specificity and 94% sensitivity) (Table 3). To classify a pool of 60 posttransplant serum samples into those

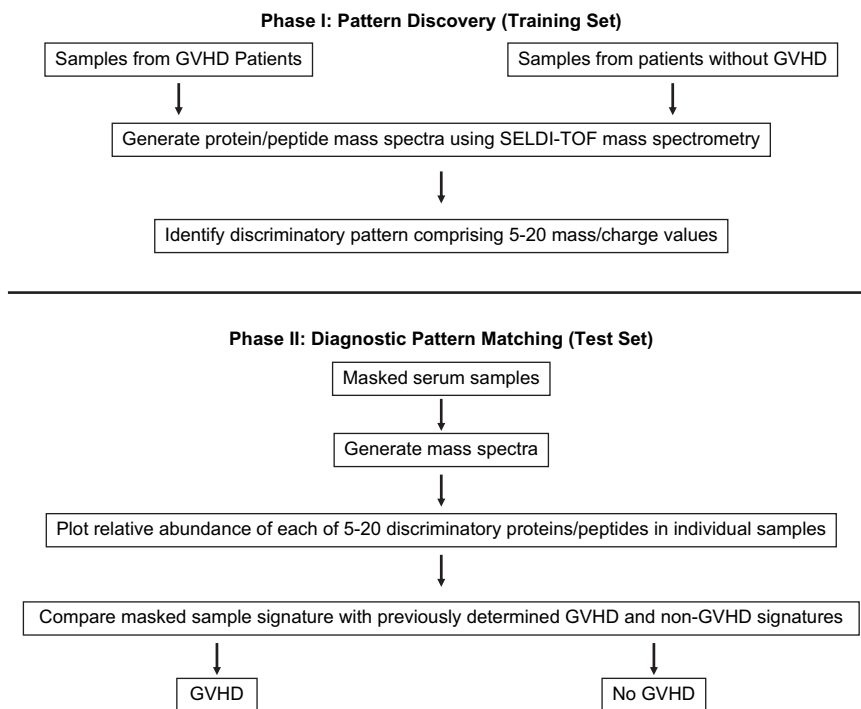


Figure 2. Scheme for analysis of proteomic spectra generated by SELDI-TOF from GVHD and non-GVHD sera.

Table 1. Summary of patient characteristics: Characteristics of patients from whom the 22 GVHD, 28 pretransplant, and 38 posttransplant non-GVHD serum samples were obtained

	GVHD (n = 22)	Non-GVHD (posttransplant) (n = 38)	Pretransplant (n = 28)	p value
Median age (range)	49 (20–65)	45.5 (26–65)	45.5 (20–59)	0.92
Sex				
Male	14	25	17	
Female	8	13	11	
Diagnosis				
Solid tumors	12 (55%)	31 (82%)	21 (75%)	
Heme malignancies	5 (22.5%)	2 (5%)	1 (3%)	
SAA/PNH	5 (22.5%)	5 (13%)	6 (22%)	
Conditioning regimen				0.55
Fludarabine + cyclophosphamide	20	37		
Other*	2	1		

SAA, severe aplastic anemia; PNH, paroxysmal nocturnal hemoglobinuria.

*One sample in each group was obtained from a patient undergoing a transplant following conditioning with TBI/cyclophosphamide/fludarabine. One sample from the GVHD group was obtained from a patient undergoing a transplant following melphalan/fludarabine conditioning.

representing GVHD or no GVHD, spectra from a “training set” of 34 posttransplant samples (12 GVHD and 22 posttransplant non-GVHD) were first analyzed. A group of 8 key ions with distinct M/Z ratios was identified that defined an optimal discriminatory pattern segregating GVHD from non-GVHD samples (Fig. 3). The GVHD-specific proteomic fingerprint represented by this 8-ion subset was not present in any of the non-GVHD samples, and when applied to 26 posttransplant blinded samples, accurately distinguished all 10 GVHD from 16 non-GVHD samples (sensitivity 100%, specificity 100%) (Table 3). Similarly, spectra from 13 GVHD and 16 pretransplant serum samples were used to generate a set of 9 M/Z ratios (discriminatory M/Z values—2986.4, 3218.2, 3818.8, 5660.7, 6489.1, 6704.2, 7026.1, 9813.0, 10883.2) that accurately identified all 21 masked GVHD (n = 9) and pretransplant (n = 12) samples (Table 3).

Discussion

Serum proteomic analysis is rapidly gaining acceptance as a valuable tool in cancer diagnostics [11–16]. A variety of mass spectrometry-based approaches are currently available for proteomic analysis. These approaches differ in the ionization method and the spectrometry instrumentation employed

as well as the data-mining software used to analyze the proteomic spectra. The mass spectrometry platform used in this study has been previously utilized to identify diagnostic serum proteomic fingerprints in patients with a variety of malignancies with a high degree of sensitivity and specificity. Furthermore, both intralaboratory and interlaboratory comparisons have demonstrated that the SELDI-based platform used here yields highly reproducible results [17].

Here we present data suggesting a potential application for this emerging technology in the diagnosis of acute GVHD. Using SELDI-TOF mass spectrometry, we generated a model based on key serum protein patterns that accurately distinguished acute GVHD from posttransplant non-GVHD samples. The discriminatory proteomic pattern appeared to be independent of other patient variables and specific for GVHD since it developed only after the onset of GVHD and was absent in the corresponding non-GVHD posttransplant samples obtained from the same patients. The ability to accurately separate pretransplant from posttransplant sera, even when both samples were obtained from the same patient, further highlights the power of this technique. A recent study by Kaiser et al. elegantly demonstrated that similar technology could be used to identify peptides and polypeptide patterns from urine samples that serve as an early indicator for the development of GVHD [18]. Our study was not designed and the sample size was too small to reliably model and predict the development of GVHD from pre-GVHD sera; among the posttransplant non-GVHD samples, only 13 belonged to patients who did not subsequently develop GVHD. In contrast, we focused largely on the feasibility of identifying serum protein signatures that could aid in the rapid and accurate diagnosis of GVHD. It is important to note that the approach described here is based on the recognition of simultaneous alterations in multiple proteins or peptides, each of which might individually be insufficiently discriminatory. The

Table 2. Summary of patient characteristics: GVHD characteristics

GVHD	Number (%) (n = 22)
Organ involved	
Skin	15 (68)
Gut	12 (55)
Liver	2 (9)
≥2 organs	7 (32)
Grade	
Grade 1–2	12 (55)
Grade 3–4	10 (45)

Table 3. Proteomic pattern analysis: Summary of data

	Total # samples	# of pretransplant samples (training/test)	# of posttransplant non-GVHD samples (training/test)	# of posttransplant GVHD samples (training/test)	Sensitivity (%)	Specificity (%)
Posttransplant no GVHD vs GVHD	60	-	22/16	12/10	100	100
Pretransplant vs GVHD	50	16/12	-	13/9	100	100
Pretransplant vs posttransplant no GVHD	66	16/12	21/17	-	94	100

identity of individual proteins or peptides that constitute the discriminatory pattern, while not essential for diagnostic purposes, may provide insight into the complex biologic and biochemical processes underlying the pathogenesis of GVHD; attempts to identify these proteins are currently underway. Although our analysis was centered on the diagnosis of GVHD, the principles of serum proteomic pattern

matching could be applied to predict both GVHD risk and GVHD outcome. One area of focus is the identification of patients who develop steroid-refractory GVHD. This group of patients does not benefit from conventional corticosteroid-based therapy, and early diagnosis could lead to the timely initiation of more effective treatment strategies and, consequently, improved outcome.

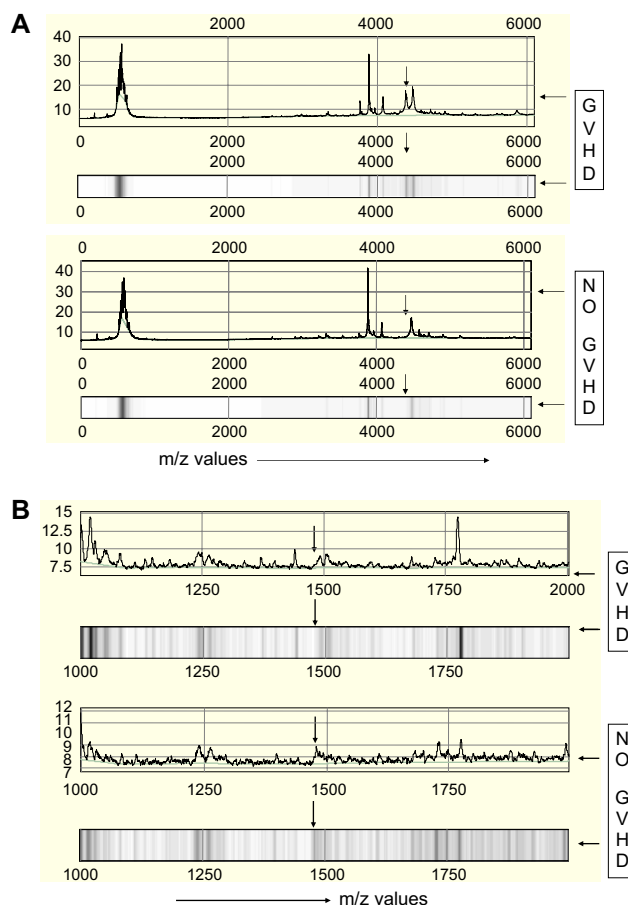


Figure 3. SELDI-TOF mass spectroscopy spectrum in serum from patients with or without acute GVHD. Representative SELDI-TOF mass spectra from patients with and without GVHD are depicted both as mass chromatograms and density plots. **(A)** The mass spectrum from a serum sample obtained at the time of onset of acute GVHD (day +106 posttransplant, upper panel) shows the appearance of a new protein peak at M/Z 4402.9 (arrow), which was not present in a posttransplant serum sample obtained several weeks before the onset of GVHD (day +28 posttransplant, lower panel). **(B)** Conversely, a protein peak with an M/Z value of 1475.5 (arrow) is present in a non-GVHD sample, but is absent in a serum sample obtained at the time of GVHD. Spectra from 12 GVHD and 22 posttransplant non-GVHD serum samples were used to identify a discriminatory signature comprising a group of 8 such unique M/Z values (M/Z values—1475.5, 3332.0, 3391.0, 3471.7, 4032.9, 4402.9, 5117.2, 5206.3) that could discriminate between the two groups.

The current study did not attempt to classify GVHD based on the organ involved or severity, nor did it attempt to differentiate GVHD from other conditions with overlapping clinical features such as drug-induced skin rash, CMV colitis, etc.; larger studies with sufficient power to address these issues are currently underway. However, the data presented here strongly suggest that serum proteomic analysis is worthy of further exploration as an accurate, inexpensive, and rapid alternative to conventional GVHD diagnostic strategies.

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