Bioinformatic Analysis of the Urine Proteome of Acute Allograft Rejection

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Abstract. The urinary proteome in health and disease attracts increasing attention because of the potential diagnostic and pathophysiologic biomarker information carried by specific excreted proteins or their constellations. This cross-sectional study aimed to analyze the urinary proteome in patients with biopsy-proven acute rejection ($n = 23$) compared with transplant recipients with stable graft function ($n = 22$) and healthy volunteers ($n = 20$) and to correlate this with clinical, morphologic, and laboratory data. Urine samples were preadsorbed on four different protein chip surfaces, and the protein composition was analyzed using a surface-enhanced laser desorption/ionization time-of-flight mass spectrometer platform. The data were analyzed using two independent approaches to sample classification. Patients who experienced acute rejection could be distinguished from stable patients with a sensitivity of 90.5 to 91.3% and a specificity of 77.2 to 83.3%, depending on the classifier used. Protein masses that were important in constructing the classification algorithms included those of mass 2003.0, 2802.6, 4756.3, 5872.4, 6990.6, 19,018.8, and 25,665.7 Da. Normal urine was distinguished from transplant urine using a protein marker of mass 78,531.2 Da with both a sensitivity and a specificity of 100%. In conclusion, (*1*) urine proteome in transplant recipients with stable graft function was significantly different from healthy control subjects, and (*2*) acute rejections were characterized by a constellation of excreted proteins. Analysis of the urinary proteome may expedite the noninvasive prediction of acute graft rejection, thus importantly assisting in establishing the diagnosis.

Acute rejection is characterized by a sudden deterioration in renal allograft function, which is routinely detected by a rise in serum creatinine in the posttransplantation monitoring clinic (1). Acute rejection may lead to graft loss either immediately or as a result of chronic allograft nephropathy (2–4). Although the current diagnostic standard rests on a set of histologic parameters on percutaneous renal allograft biopsy, a plethora of additional markers of acute rejection, from clinical symptoms to the genome-wide analysis of biopsy specimens, have been described (5,6). There is growing consensus that such a complex and heterogeneous process could best be fingerprinted using not a single biomarker but rather a constellation of individually informative biomarkers (6).

Proteome-wide searches for biomarkers of disease processes have already proved valuable in diagnosing bladder tumors and breast and ovarian cancer (7–9). Similarly, urinary biomarker identification using proteome-wide analysis may provide an unbiased noninvasive and expeditious diagnosis of allograft dysfunction. Although any biologic fluid may be examined in search of biomarkers, the overriding principle is the simplicity of obtaining such a material for diagnostic purposes. In the case of acute graft rejection, the obvious choice of diagnostic material is the urine, a biologic fluid that potentially is most reflective of pathologic events that take place in the kidney. Indeed, previous genomic studies revealed differential expression of several gene markers in inflammatory cells excreted with the urine (10). One of the requirements for analytic platforms that are used for identification of biomarkers is high throughput. Currently, this requirement is best met by the existing surface-enhanced laser desorption/ionization time-of-flight (SELDI-TOF) mass spectrometer system. Previous studies have demonstrated that this system for biomarkers discovery offers high sensitivity (93 to 100%) and specificity (87 to 99%) and positive predictive value of 84 to 99% (7,9), yet the day-to-day analysis using this approach is far from being

Received March 15, 2004. Accepted August 27, 2004.
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1046-6673/1512-3240
Journal of the American Society of Nephrology

DOI: 10.1097/01.ASN.0000145241.83482.68

routine, requiring technical precision followed by exhaustive bioinformatic analysis. The main thrust of the latter analyses is to select the optimal number of the most valuable variables—protein peaks—which will have the highest discriminative and predictive power. Although several bioinformatic approaches have been adapted for this purpose, none is faultless or universally superior to others. It follows, therefore, that several analytic bioinformatic approaches need to be entertained when searching for an algorithm that discerns one pathologic process from the others (11).

Two separate investigative groups recently described changes in urinary protein excretion in transplant patients with biopsy-proven acute rejection (12,13). Intriguing is that no common findings have been reported. It is possible that the disparate findings of two independent studies of the same pathologic process in different patient populations could in part reflect the paucity of bioinformatic analyses and/or differences in patient population. In either case, the validity of biomarkers would be diminished. For this reason, we used multiple bioinformatic approaches to studies of acute rejection in three different centers that use individual therapeutic maintenance regimens, with the aim of detecting universal classifiers that eventually could serve as biomarkers of this pathologic process. The aim of the present study was to characterize the urinary proteome of acute rejection compared with patients with stable allograft function and to compare two approaches to the analysis of SELDI-TOF data.

Materials and Methods

Patients

All kidney transplant patients who were followed in three ethnically and immunosuppressive protocol distinct Medical centers (Westchester Medical Center, Charite, Campus Virchow-Clinic, Berlin, and Munich University Medical Center) were eligible for inclusion in the study. Urine was collected at routine follow-up in the outpatient clinic or at time of diagnosis of acute rejection.

Clinical data recorded included urinalysis, BP, serum creatinine, and levels of immunosuppressant agents. All patients completed a consent form for urine collection and for release of clinical data in compliance with Health Insurance Portability and Accountability Act regulations, and the protocols were approved by each of the Institutional Review Boards for studies involving human subjects.

Only those who were stable, with no acute medical problems, were included in the baseline group ($n = 22$). Renal histology, when obtained at time of transplant ($n = 12$), showed no significant pathology. The rejection group represents those who had a biopsy-proven acute rejection during follow-up ($n = 23$). All transplant biopsies were reviewed by renal pathologists and scored according to the BANFF criteria (5).

Standard maintenance immunosuppression included a calcineurin inhibitor (tacrolimus [trough level 5 to 10 ng/ml], or cyclosporin [trough level 50 to 150 ng/ml]), rapamycin (trough level 10 to 15 ng/ml), and prednisone in tapering doses from 120 to 10 mg/d at 4 wk after surgery. Control samples were obtained from 20 healthy volunteers.

Sample Processing

Fresh urine samples were obtained in the clinic and immediately placed on ice to prevent degradation by endogenous proteases. These were subsequently centrifuged at 2500 rpm for 10 min at 4°C and stored at −80°C. Protease inhibitors were not used to avoid possible ion suppression of endogenous proteins. In preliminary studies, four paired urine samples were analyzed with varied storage times, and it was demonstrated that the proteome remained stable for at least 4 h on ice (data not shown). Similar results on urine storage were reported by Schaub *et al.* (14)

Mass Spectrometry Analysis of Urine on Protein Chips

Before analysis, the urine samples were thawed on ice, and 160 μl of urine was added to 60 μl of denaturing buffer that contained 9 M urea, 2% CHAPS, and 50 mM Tris (pH 9) (15). Twenty-five microliters of this denatured urine solution was then added to a bioprocessor, a 96-well array in which the base of each well is formed by the chip surface, and incubated with 100 μl of binding buffer overnight. The binding buffer constituents are crucial to the protein adsorption to the chip surface and are detailed in Table 1. The wells then were aspirated dry and washed three times. Saturated sinapic acid 0.5 μl, the energy-absorbing molecule that enhances transformation of proteins to the gaseous phase, was added twice and allowed to dry.

Table 1. Conditions and binding buffers used for the different chip surfaces

Mass range	Chip Type							
	CM10		Q10		H50		IMAC-30	
	Low	High	Low	High	Low	High	Low	High
Maximum molecular mass	100	200	100	200	100	200	100	200
Optimized range	2–20	20–50	2–20	20–50	2–20	20–50	2–20	20–50
Laser intensity	197	200	197	200	192	200	192	200
Detector sensitivity	7	7	7	7	7	7	7	7
Laser shots per spot	130	130	130	130	130	130	130	130
Deflector mass (kDa)	2	50	2	50	2	50	2	50
Detector voltage	2850	2850	2850	2850	2850	2850	2850	2850
Buffer used	100 mM Na acetate (pH 4.0)		50 mM TrisHCL (pH 9.0)		30% Acetonitrile, 1 mM HEPES		0.2 M NACL in PBS	

We used four different types of chips to ensure that the panoply of protein types were adsorbed. Protein chips used had the following characteristics for enhancement of the absorption and detection of proteins with different properties: H50 (hydrophobic surface), Q10 (strong anion exchange), CM10 (weak cation exchange), and IMAC30 (immobilized metal affinity capture).

After the energy-absorbing molecule had dried, chips were loaded into a SELDI-TOF mass spectrometer and the samples analyzed by laser ionization of the proteins from the chip. We averaged the results of 130 shots per spot. The spot protocol was kept constant throughout the study. The laser intensity, the sensitivity, and the detector voltage determine this protocol known as a "reading condition." The protein/matrix was energized using different conditions for low molecular weight (<27,000 Da) and high molecular weight species (27,000 to 200,000 Da). We have previously demonstrated that the accuracy of the molecular weight estimation is within 0.5% for low molecular weight proteins, and the detection sensitivity is at least 0.1 fmol at this range (16).

External calibration was performed using all-in-one-peptide standard for low molecular weight and all-in-one-protein for higher molecular weights (Ciphergen, Freemont, CA). All of the samples were analyzed in a single batch using the same calibration equation. Peak detection was performed using the proprietary Ciphergen software using a filter setting of 0.2 times the expected peak and a peak definition of signal ≥4 times the noise. Baseline subtraction was performed on all spectra, and the data were normalized using total ion current normalization for spectral comparison (17).

Approaches to Data Analysis

Two different bioinformatic approaches to data analysis were used. The biomarker pattern software (BPS; Ciphergen) is a proprietary software that is based on the data mining software Classification and Regression Trees. This classifies cases by applying a "rule" determined by the peak intensity (height) of a particular protein mass. This is advantageous for classification by a minimum number of proteins, but classification performance is inferior to algorithms that use multiple trees and more protein masses. The data are analyzed using 10-fold cross-validation, *i.e.*, a model is constructed on 90% of the data and then tested on the remaining 10%. This then is performed 10 times. Second, data were analyzed using Random Forest, available as part of the R. gnu software suite, which uses multiple-tree analysis to classify the samples into groups as described by Breiman *et al.* (18,19). It creates trees by classifying the cases according to a user-defined set number of protein peak intensities. Each tree then "votes" for inclusion of an individual case in a particular group. We used 22 protein peaks at each branching point and created 5000 trees at each analysis. The number of variables used at each branch point was chosen using the Rftune function that plots the number of variables against the classification error, and the variable number is chosen to minimize the error. The importance of any particular protein mass is calculated by randomly permuting all of the values for a particular protein mass and running the new values down a tree. The measure of importance of the variable is the average reduction in the accuracy of the class votes. In this form of analysis, the data are split into two groups with ~33% of the data acting as a test set or "out of bag."

Differences in mean arterial BP and serum creatinine between groups were analyzed using the Mann-Whitney *U* test for nonparametric data. A repeated measures ANOVA was used to measure change in the serum creatinine in the stable patients. The Kolmogorov-Smirnov (K-S) test was used to assess normality. Results are presented as mean ± SD.

Results

General Characteristics of the Study Population and the Pool of Urine Proteins

Urine samples from 22 stable patients characterized by a steady serum creatinine for at least 4 wk before and after sample collection were analyzed. All stable patients analyzed received their allograft within the previous 12 mo (mean, 102.9 ± 115.5 d). Mean creatinine stabilized at 1.6 ± 0.7 mg/dl after transplantation was 1.6 ± 0.6 at time of urine sampling and was 1.6 ± 0.5 at last follow-up in the stable patient group ($P = 0.5$ repeated measures ANOVA, K-S = 0.6). Mean follow-up after sampling was 133.2 ± 136.6 d. Urine samples from patients with acute rejection were obtained before treatment for acute rejection. Mean creatinine in this group was 2.6 ± 2.7 mg/dl (not significantly different from the stable group, $P = 0.5$, Mann-Whitney *U* test, K-S = 0.001). Transplant patients included were maintained on the immunosuppressant treatments outlined in Table 2.

We identified a total of 425 protein peaks after SELDI analysis of all samples. A composite peak map illustrating all urine samples from each group is shown in Figure 1. Normal urine and stable transplant and acute rejection urines contained a sum of 292, 365, and 340 protein peaks, respectively.

As an initial analysis, we plotted all 65 subjects in multiple dimensions using all 425 protein peaks identified to visualize the data distribution. This multidimensional scaling plot is a device that reveals structure or groups in the data. It is derived from the Random Forest generated proximity matrix, where the (i, j) element of the proximity matrix is the fraction of trees in which elements i and j fall in the same terminal node and demonstrated clearly that proteomic differences existed between the three groups: controls, stable transplant, and acute rejection (Figure 2). This finding justified further data mining.

Urinary Proteome of Healthy Volunteers Compared with Stable Transplant Recipients

Clinical utility of biomarkers dictates that the detection of a pathologic process involves a handful of proteins at most.

Table 2. Patient profiles[a]

	Group		*P* Value
	Stable ($n = 22$)	Acute Rejection ($n = 23$)	
Gender (M:F)	15:7	19:7	—
Immunosuppression			
tacrolimus	15/22	17/23	
cyclosporin	7/22	4/23	
rapamycin	20/22	15/23	
steroid	22/22	23/23	
mycophenolate	2/22	8/23	
BP (MAP)	98.2 ± 11.4	96.6 ± 10.4	NS
Serum creatinine (mg/dl)	1.6 ± 0.6 Vs	2.6 ± 2.7	NS

[a] MAP, mean arterial pressure.

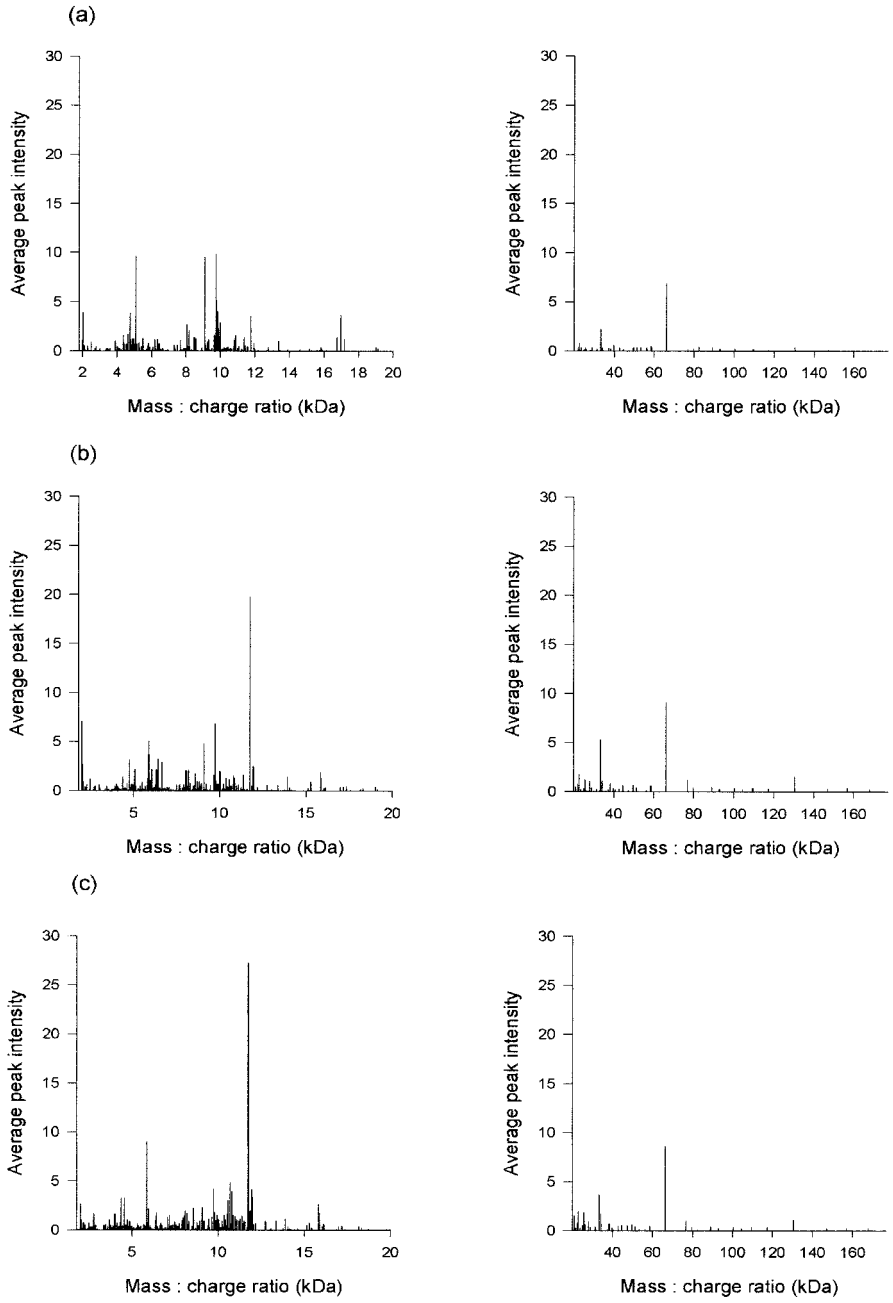

Figure 1. A chart representing surface-enhanced laser desorption/ionization time-of-flight–detected peaks. (A) Healthy volunteers. (B) Stable transplant recipients. (C) Patients with acute rejection.

Therefore, the BPS then was applied as outlined in the Materials and Methods section. The advantage of this analysis is that it demonstrates the accuracy with which few proteins can characterize different conditions (Figure 3A). Indeed, stable transplant patients could be distinguished accurately (100% predictive value) from the control population on the basis of a splitting rule involving only one protein of molecular mass 78,531.2 Da (Table 3).

Further analysis was performed using the Random Forest program to determine whether the patients could be classified correctly on the basis of the protein mass data and to identify the constellation of important proteins on the basis of their molecular mass. This analysis had excellent predictive ability with no detectable error in either the training or the test sets (Table 3).

Stable patients and healthy volunteers then were compared to determine which protein peak masses are most valuable in fingerprinting the urine of stable transplant recipients. The Random Forest variable importance plot (Figure 3B) shows the molecular mass of the most valuable variables in descending order. These results indicated that partitioning the groups accurately on the basis of these protein mass peak data are feasible using a large number of proteins.

Patients Who Experienced Acute Rejection versus Stable Patients

The acute rejection patients then were compared with the stable patients, and similar analyses were performed. The BPS analysis showed that the most important of these biomarkers as

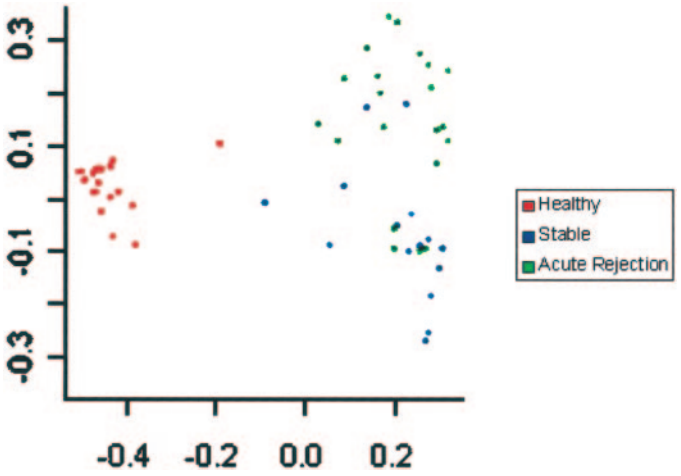

Figure 2. Multidimensional scaling plot of control, stable patients and patients with acute rejection. The scales represent the proximities of different protein peaks.

assessed by a single-tree analysis had molecular weights of 4756.3, 6990.6, 2802.6, and 2003.0 Da (Figure 4A).

The Random Forest generated variable importance plot is shown in Figure 4B. A protein of mass 4756.3 again was identified as the most valuable. The classification precision for BPS and Random Forest is presented in Table 4. This shows a sensitivity ranging from 90.5 to 91.3% and a specificity ranging from 77.2 to 83.3%.

Discussion

The data presented herein illustrate a broad-based, unbiased, noninvasive proteomic and bioinformatic approach to the diagnostic problem of acute allograft rejection. We analyzed samples from different countries to avoid any center or immunosuppressive protocol–dependent effect and used four available ProteinChip surfaces to capture a maximal number of proteins. The large quantity of protein data generated required complementary bioinformatic tools to identify diagnostically valuable patterns, and we used two different mathematic apparatus to arrive at the overlapping set of results.

Although acute rejection rates have fallen since the introduction of newer immunosuppressant regimens with rates declining from 43.3 to 23.9% for cadaveric and from 35.8 to 23.4% for living kidney donors between 1988 and 1996, maximizing graft longevity remains a priority (3). Acute rejection continues to be a feared complication of transplantation not only because of the resultant direct morbidity and necessary increase in immunosuppression but more important because of its deleterious effect on graft survival. Hariharan *et al.* and other investigators (3,20) noted that the absence of acute rejection during the first posttransplantation year resulted in a predicted increase in graft half-life from 8.8 to 17.9 yr for cadaveric renal allografts performed in 1995. Oyo *et al.* (21) reported that acute rejection was associated with an increased risk of death with a functioning graft, and Kaplan *et al.* (22) noted that acute rejection, as a cause of graft loss, was associated with reduced survival on returning to dialysis. The survival of patients with a functioning transplant is higher than those on dialysis and on the transplant waiting list (23). Their survival is also higher than those who return to dialysis after a graft has failed (22). Currently, return to dialysis after graft loss is an important cause of entering a dialysis program, accounting for 4.7% in the United States in 2001 (24).

These epidemiologic data highlight the requirement for further improvements in diagnosing and combating acute rejection. Currently, an elevation of serum creatinine is routinely used as a biomarker for acute rejection (1). Once an elevation in serum creatinine is detected, a biopsy is procured to exclude alternative diagnoses. A Banff graded, two-core allograft biopsy remains the diagnostic gold standard. Unfortunately, serum creatinine is not a sensitive biomarker. It has been shown that up to 30% of grafts with "stable" creatinine may have smoldering rejection and that treatment of this subclinical rejection may result in improved graft function (4,25). Neither is there a relationship between creatinine peak and the severity of rejection (26). Serum creatinine may become elevated only some time after immune activation in the graft, and the length of time during which the creatinine is elevated may have a deleterious effect on the graft (20,27,28). In fact, our data are in agreement with this thesis: Despite biopsy-proven rejection, there was no significant difference in serum creatinine between these patients and those with stable graft function. A more precipitous and sensitive yet specific biomarker, therefore, is warranted. The search for surrogate early biomarkers of rejection to supplement renal allograft biopsy remains a challenge. Their characterization would make the diagnosis of rejection more expedient and noninvasive. Ideally, new biomarkers would also provide greater sensitivity. Diverse approaches have been entertained to identify biomarkers for acute rejection, including urinary cytokine estimation, urinary complement levels, urine cytology, urine zymology, urine lymphocyte receptor detection, blood cytokine estimation, blood lymphocyte receptor expression, and various blood enzyme assays. Radiologic techniques have also been proposed (29). None of these has proved to be definitive. Recent advances in mass spectrometry may help to make the goal of urinary diagnosis achievable. Precedents of their diagnostic potential in detecting bladder carcinoma and ovarian cancer exist (7,8).

The analysis of this type of data is particularly complex because (*1*) data are obtained with large numbers of variables for each patient, (*2*) even small detection errors can have a large effect, (*3*) the usual proprietary statistical programs have limitations in dealing with a large number of variables, and (*4*) each analytical approach has its advantages and weaknesses. For this reason, we used two different types of data-mining techniques. In particular, a protein of molecular mass 78.5 seems to be important in the characterization of stable transplant urine. Proteins of mass 2003.0, 2802.6, 4756.3, 5872.4, 6990.6, 19,018.8, and 25665.7 Da seem to be important in distinguishing patients with acute rejection from stable patients. This differs from the results of Clarke *et al.* (12), who found that proteins of 6.5, 6.6, 6.7, 7.1, and 13.4 kD performed well as biomarkers of acute rejection. We found a protein of peak mass 13.4 kD to be a reasonably good discriminator of

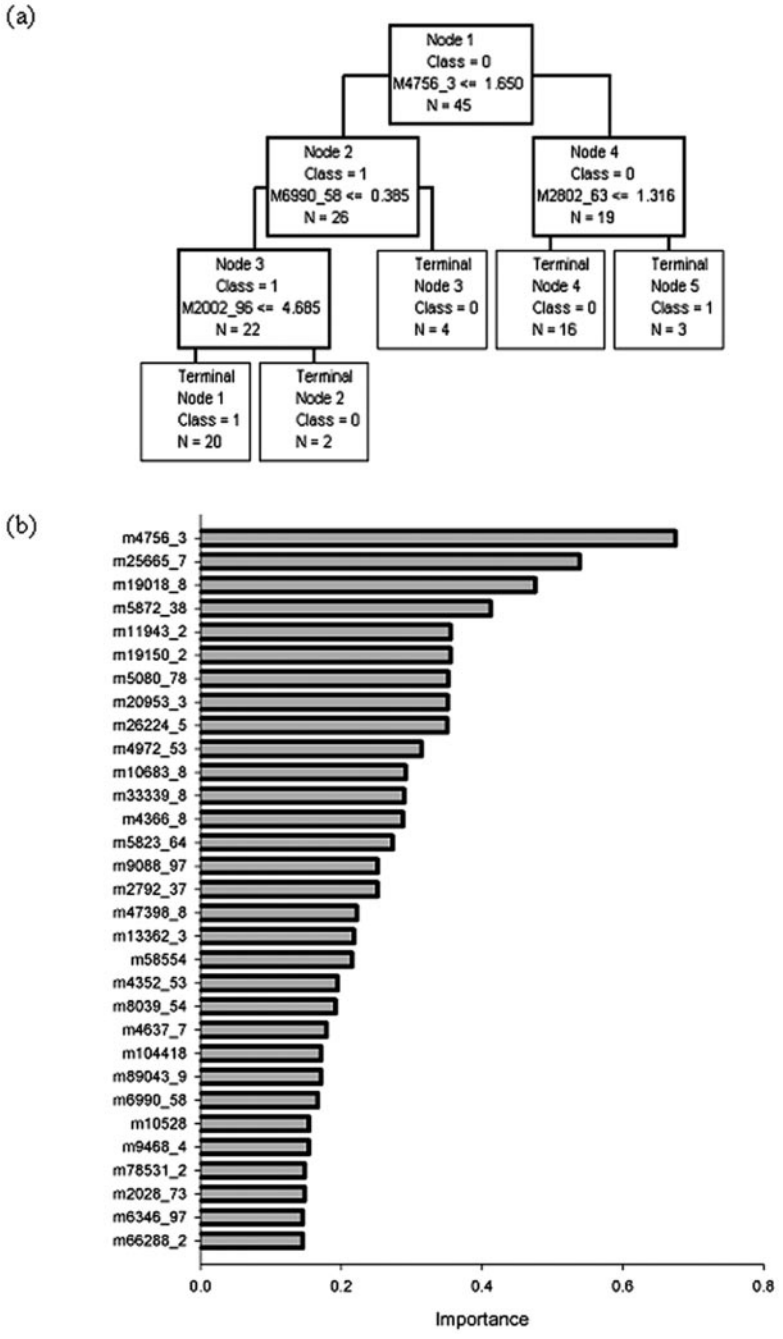

Figure 3. Comparison of healthy volunteers and stable transplant recipients. The biomarker pattern software (BPS) analysis shows the accuracy of division using only the best single classifier, *ie.* a protein peak of a certain intensity. Class 0 = healthy volunteers and class 1 = stable transplants (A). Protein peaks identified as valuable discriminators by the Random Forest package (B).

Table 3. Classification precision for BPS and Random Forest for stable transplant patients compared with healthy volunteers

	Healthy Volunteers	Stable Patients	Sensitivity	Specificity	Positive Predictive Value	Negative Predictive Value
BPS						
healthy volunteers (n = 20)	20	0	100%	100%	100%	100%
stable patients (n = 22)	0	22				
Random Forest						
healthy volunteers (n = 20)	20	0	100%	100%	100%	100%
stable patients (n = 22)	0	22				

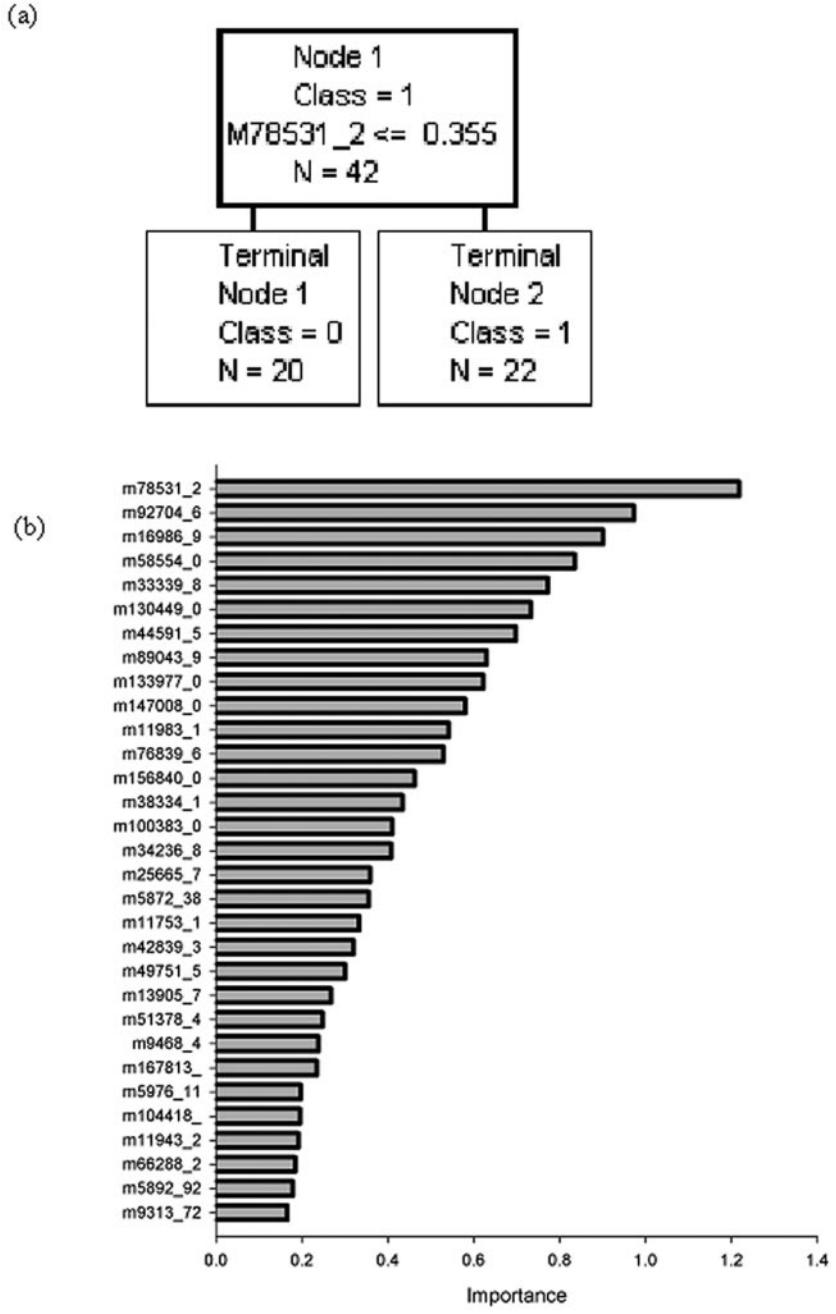

Figure 4. Comparison of patients who experienced acute rejection with stable transplant recipients. The BPS analysis shows the accuracy of division using only the best single classifier, *ie.* a protein peak of a certain intensity. Class 0 = stable transplants and class 1 = acute rejection (A). Protein peaks identified as valuable discriminators by the Random Forest package (B).

acute rejection but not the most valuable. Schaub *et al.* (13) found proteins of mass ranging from 5270 to 5550, 7050 to 7360, and 10,530 to 11,000 Da to be the best discriminators, which differs from our findings. These differences may suggest that the results of SELDI are erratic. It must be remembered that there are thousands of proteins in the urine, and the use of different chip surfaces will result in the adsorption of different proteins to the chip surface with the remainder of the proteome being discarded. This study is the first to use four different types of chip surface to detect a wide variety of proteins. The studies by Clarke *et al.* used IMAC-3 and H4 chips (equivalent to the IMAC30 and H50 chips used in this study), and Schaub *et al.* used NP20. It is also important to note that the technology continues to advance and that the chip surfaces available today are different from previous chip generations. Furthermore, the proteins reported in a paper would be only a small fraction of those detected, and as there are many possible biomarkers in the urine, those reported as giving the best results by one group will not necessarily preclude the validity of biomarkers reported by different investigators. Differences in peak detection and data analysis can also result in different biomarker reporting from the same data set. Schaub *et al.* used a signal ratio of ≥ 3, whereas we used a signal noise ratio of ≥ 4. The signal noise ratio used by Clarke *et al.* is unstated. It is also possible that different immunosuppression regimens or differences in proportion of cadaveric *versus* live donors may result in proteome alteration.

The lack of protocol biopsies is a weakness in our study; it

Table 4. Classification precision for BPS and Random Forest for stable transplant patients compared with patients who experienced acute rejection

	Acute Rejection	Stable Patients	Sensitivity	Specificity	Positive Predictive Value	Negative Predictive Value
BPS						
acute rejection ($n = 23$)	21	5	91.3%	77.2%	80.7%	89.5%
stable patients ($n = 22$)	2	17				
Random Forest						
acute rejection ($n = 23$)	19	4	90.5%	83.3%	82.6%	90.9%
stable patients ($n = 22$)	2	20				

is possible that some of the stable transplant patients have ongoing subclinical acute rejection or an unknown degree of chronic damage. Stability of their renal function before and after and urine sampling and good discrimination from acute rejection are arguments for the homogeneity of the stable group. The excellent predictive power of these bioinformatic tools in distinguishing healthy patients from those with a stable renal allograft was less accurate in distinguishing stable patients from those with acute rejection, indicating that noise in the classifying variable is a possibility. These drawbacks notwithstanding, the resultant accuracy is comparable to the most discriminating tests.

Further work remains to be done to test the proposed diagnostic patterns in a larger and even more ethnically diverse patient population and, if confirmed, to chemically identify each protein peak in an attempt to convert a relatively sophisticated procedure into a bedside test of higher clinical utility.

Acknowledgments

These studies were supported in part by National Institutes of Health Grants DK45462, DK54602, and DK52783 (to M.S.G.) and by the Westchester Artificial Kidney Foundation. E.O.R. was supported by a Kevin J. and Gloria B. Keily National Kidney Foundation Fellowship during this study.

We acknowledge the help of the biochemical laboratory at Westchester Medical Center, especially Jacqueline McKenzie and Marlene Williams. We thank Prof. Petra Reinke, Division of Nephrology and Intensive Medical Care, Charite, Campus Virchow-Clinic-University Medicine Berlin for outstanding help in recruiting patients for this study.

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