Proteomic Analysis of Urine in Kidney Transplant Patients with BK Virus Nephropathy

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The differentiation of BK virus–associated renal allograft nephropathy (BKVAN) from acute allograft rejection (AR) in renal transplant recipients is an important clinical problem because the treatment can be diametrically opposite for the two conditions. The aim of this discovery-phase biomarker development study was to examine feasibility of developing a noninvasive method to differentiate BKVAN from AR. Surface-enhanced laser desorption/ionization (SELDI) time-of-flight mass spectrometry analysis was used to compare proteomic profiles of urine samples of 21 patients with BKVAN, 28 patients with AR (Banff Ia to IIb), and 29 patients with stable graft function. SELDI analysis showed proteomic profiles that were significantly different in the BKVAN group *versus* the AR and stable transplant groups. Peaks that corresponded to m/z values of 5.872, 11.311, 11.929, 12.727, and 13.349 kD were significantly higher in patients with BKVAN. Bioinformatics analyses allowed distinction of profiles of patients with BKVAN from patients with AR and stable patients. SELDI profiles also showed a high degree of reproducibility. Proteomic analysis of urine may offer a noninvasive way to differentiate BKVAN from AR in clinical practice. The identification of individual proteomic peaks can improve further the clinical utility of this screening method.

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BK virus (BKV) infection is common in the general population (1,2). The importance of BKV infection in healthy individuals is unclear, but it has emerged to be a significant problem in immunocompromised patients, such as bone marrow transplant patients and patients with solid-organ allograft (1–3). BKV renal allograft nephropathy (BKVAN) now is recognized to have an important role in development of renal allograft dysfunction (3–6). In recent studies, the prevalence of asymptomatic BK viruria in kidney transplant patients has been reported to be on the order of 30% (7). Approximately 6 to 10% of these patients develop BKVAN, and the reported graft loss rate in this group has been as high as 50% (6,7).

BKVAN can resemble acute allograft rejection (AR) both clinically and histologically (3–5). Differentiation between BKVAN and AR is important, however, because the treatment is diametrically opposite for the two conditions. In general, immunosuppression needs to be reduced in patients with BKVAN, whereas it is increased in AR. Currently, these two clinical conditions cannot be differentiated in a reliable way on the basis of clinical and laboratory findings, and a definitive diagnosis of BKVAN requires allograft biopsy. Even the histologic differentiation of BKVAN from AR can be difficult unless viral inclusions are seen on allograft biopsy (4,5).

Early detection of patients with increased risk for BKVAN is likely to improve their ultimate outcome. However, currently, no noninvasive methods are available for this purpose. During the past few years, proteomic profiling of blood and urine samples has been used to develop noninvasive biomarkers for several pathologic states, including various cancers (8–12). Recently, such techniques also have been used in developing diagnostic algorithms for AR in kidney transplant patients (13–17). In this study, we report that proteomic analysis of urine potentially may be a noninvasive way to differentiate patients with BKVAN from patients with stable graft function or AR.

Materials and Methods

These studies were reviewed and approved by Institutional Review Boards of the University of Pittsburgh Medical Center and Children's Hospital of Pittsburgh.

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Table 1. Patient characteristics[a]

Characteristic	Stable ($n = 29$)	BKVAN ($n = 21$)	AR ($n = 28$)
Female gender (%)	15 (52)	6 (29)	10 (36)
Age (mean [range])	53 (21 to 73)	51 (23 to 78)	45 (19 to 68)
White race (n [%])	26 (90)	18 (86)	25 (89)
Primary diagnosis (n [%])			
diabetic nephropathy	7 (24)	6 (29)	4 (14.3)
glomerulonephritis	6 (21)	4 (19)	9 (32)
polycystic kidney	7 (24)	2 (9.5)	4 (14.3)
other	9 (31)	9 (42.5)	11 (39.3)
Serum creatinine (mg/dl; mean [range])	1.2 (0.6 to 1.8)[b]	2.8 (1.7 to 4.3)	3.8 (1.6 to 13.1)
Cadaveric donor (n [%])	14 (48)	17 (81)	19 (68)
Transplant number (median)	1	1	1
No. of rejections (median [range])	0	2 (1 to 5)	1 (1 to 4)
Months posttransplantation (mean [range])	9.3 (1 to 60)[c]	13.9 (1 to 54)	21.2 (1 to 77)

[a]AR, allograft rejection; BKVAN, BK virus–associated renal allograft nephropathy.
[b]$P < 0.001$ *versus* BKVAN and acute rejection groups.
[c]$P = 0.002$ *versus* AR group.

Patients and Sample Collection

Urine and blood samples were collected from all kidney transplant patients at the University of Pittsburgh Medical Center who consented. As a part of the study protocol, the initial samples were obtained within 48 h after transplantation and thereafter during each routine follow-up visit. Patients who were undergoing kidney biopsy also provided a voided urine sample <24 h before the biopsy. One portion of each sample was sent for routine examination, and an aliquot was centrifuged at 2000 rpm for 10 min at 4°C and stored at −80°C until further analyses without any protease inhibitors.

Kidney transplant patients with biopsy-proven BKVAN and their clinical information were retrieved from an electronic database. Urine samples were available from 21 of these patients. Further searches were performed to identify the two comparison groups of 28 patients with AR and 29 patients with stable graft function. Acute and chronic AR was classified according to the Banff classification (18).

Detection of Viral Infections

Each study patient was screened for BKV viruria and viremia using a quantitative PCR assay (19). BKVAN was diagnosed by histopathologic examination, which typically showed viral inclusions and positive immunohistochemistry and/or *in situ* hybridization (19). Urine samples additionally were screened for cytomegalovirus (CMV), adenovirus, and human herpesvirus 6 (HHV-6) using PCR assays. CMV screening also was performed by detection of pp65 antigenemia.

Surface-Enhanced Laser Desorption/Ionization Time-of-Flight Mass Spectrometry Analyses

Urine samples were thawed and centrifuged at 15,000 × *g* for 5 min at 4°C. Protein content was measured using the Bradford assay. Urine sample aliquots totaling 10 μg of protein were dispensed, adjust to 160 μl of final volume with PBS, then flash-frozen again and stored at −80°C before use on surface-enhanced laser desorption/ionization (SELDI) chips. Samples were analyzed using two chip types (IMAC30 and CM10) on three replicate spots, with total protein of roughly 1.7 μg per spot.

The IMAC30 ProteinChip arrays were preactivated using Ciphergen Bioprocessor (Ciphergen Biosystems, Fremont, CA) by loading with 50 μl of 100 mM $CuSO_4$ on each spot. The chips were shaken on a tube mixer (Tomy Seiko Co., Ltd., Tokyo, Japan) for 5 min (speed form 20, amplitude 7). Each array spot was rinsed with 200 μl of HPLC-grade water and aspirated before addition of 50 μl of sodium acetate (100 mM, pH 4). The chips then were equilibrated twice for 5 min with 200 μl of binding buffer (PBS + 0.2 M NaCl). For preactivation of CM10 ProteinChip, the arrays were equilibrated for 5 min with 200 μl of binding buffer (100 mM sodium acetate, pH 4).

The urine samples were thawed on ice and denatured with 60 μl of 9 M Urea/4% CHAPS in 40 mM Tris (pH 9) and then further diluted 1:5 in IMAC30 and CM10 binding buffers. The samples were arrayed in a blinded layout of combined case/control samples, together with a standard pooled sample (one spot on each array for quality assurance purposes). Arrays were incubated overnight with shaking at 4°C, then rinsed twice for 5 min with binding buffer, then twice for 1 min with water, and air-dried for 20 min. Energy-absorbing matrix (sinapinic acid) in 50% acetonitrile/0.5% trifluoroacetic acid was applied to arrays using automated rapid spotting on Biomek2000 robotic workstation

Table 2. Renal allograft biopsy rejection scores (Banff classification)

Rejection Scores	BKVAN ($n = 21$)	AR ($n = 28$)
Acute		
0	6	0
IA	5	13
IB	9	12
IIA	1	2
IIB	0	1
Chronic		
0	6	7
IA	8	16
IB	0	0
IIA	5	5
IIB	2	0

(Beckman-Coulter, Fullerton, CA) in two 1-μl applications, separated by 5 min. Chips were air-dried for 1 h and stored in the dark at room temperature until SELDI analysis.

The reacted ProteinChip Arrays were analyzed using the PBSIIc ChipReader mass spectrometer. The spotted chips were read, in random order, in an uninterrupted run using the Ciphergen ChipReader AutoLoader device. The SELDI time-of-flight mass spectrometry (SELDI-TOF-MS) spectra (0 to 100 kD) were collected by the accumulation and averaging of 192 laser shots from 16 positions across the width of the ProteinChip Array spot. A laser intensity of 180 was used in a positive ion mode, ensuring that transient shot intensities were below saturation of the detector, with a detector sensitivity setting of 9 (2900 V) and a focus lag time of 900 ns, using mass deflection at 1500 Da. The protein masses were calibrated externally using the 7-in-1 purified peptide molecular mass standard (Ciphergen Biosystems).

Data Processing and Statistical Analysis

The spectra that were generated by SELDI-MS-TOF were analyzed using two independent software tools: (*1*) Ciphergen ProteinChip with CiphergenExpress and (*2*) proteomic data analysis package (PDAP). PDAP is a data analysis program developed at the Computer Science department of the University of Pittsburgh and implemented in MATLAB (MathWorks, Natick, MA) (20).

Before analyses, all spectra were preprocessed using the following preprocessing steps: Variance stabilization, baseline correction, calibration/rescaling, smoothing, and profile alignment. Spectra with the total ion current normalization factor of >2 SD over the mean were considered bad spots and excluded from the subsequent analyses. After preprocessing, profile peaks were identified. All subsequent data analysis was performed on these peaks.

Univariate Analysis

The three patient groups were compared for demographic and other clinical characteristics using the *t* test for continuous variables and χ^2 test for categorical variables. For the SELDI data, Ciphergen ProteinChip software was used to detect peaks, and the corresponding peak intensities were exported to Microsoft Excel. The significance of differentially expressed peaks among three groups was studied using the Kruskal-Wallis one-way ANOVA on ranks. Pairwise comparisons between groups that were found to be significantly different on Kruskal-Wallis test were done using *t* test (SPSS 13.0 statistical software; SPSS, Chicago, IL).

Multivariate Analysis

The discriminative potential of peak combinations in proteomic profiles was analyzed using multivariate statistical prediction models. Three prediction tasks were considered in multivariate analyses: (*1*) BKVAN *versus* AR, (*2*) BKVAN *versus* stable transplant, and (*3*) AR *versus* stable transplant. Evaluation of prediction performance was conducted using repeated random subsampling of the data in the study into multiple training and test subsets (21).

Statistical machine learning algorithms were used to evaluate the ability of profile features (peaks) to discriminate between samples that belonged to the various patient groups. Three classification algorithms were examined for potential disease prediction models: (*1*) Support vector machine (SVM) (22), (*2*) Classification and Regression Tree (CART) (23), and (*3*) Random Forest (RF) (24). All three models were trained on peaks that were identified by the PDAP program (20). In addition, the SVM algorithm was applied to the subset of the top 100 differential peaks according to the *P* value of the *t* test (25).

Each learning model was evaluated by dividing the data multiple times into training and test sets using the repeated random subsampling approach (21) with 70:30 training/testing splits. The classification model was always learned on the training set and evaluated on the test set. The performance statistics that were used to evaluate the learning models were Achieved Classification Error (ACE), sensitivity, and specificity of the model on test sets. The performance statistics of each model were obtained by averaging the results over multiple train/test divisions. The CART model in the Ciphergen-based software was evaluated on three training/testing splits. All other classification methods were evaluated on 40 different training/testing data splits.

Given the small size of the study samples, an analysis was conducted to test whether the classification results might represent a finding that may occur by chance (26). The analyses, called Permutation Achieved Classification Error (PACE), evaluates whether the classification performance that is observed on the true data could be achievable on randomly regrouped data (27). Briefly, PACE computes the empirical distribution of classification errors under null random conditions and compares it with the error on the original data. In each permutation, a sample is randomly reassigned to one of the disease categories

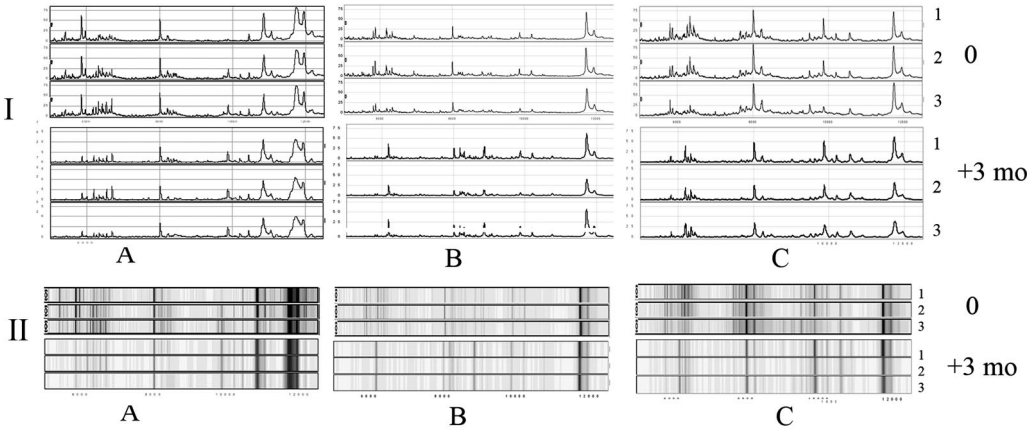

Figure 1. Surface-enhanced laser desorption/ionization (SELDI) analysis of urine samples from patients with BK virus–associated renal allograft nephropathy (BKVAN; A), patients with allograft rejection (AR; B), and patients with stable graft function (C) analyzed in triplicate (1 through 3) at onset of study (O) and the same samples analyzed on separate experiment 3 mo later (+3). Proteomic profile of the samples is shown as peak intensities (I) and as false-gel image (II).

(BKVAN, AR, or control). For example, the sample (and its profile) that belonged to a patient with BKVAN was randomly reassigned to either the AR, the control, or the BKVAN group. This process was repeated for each sample until all 78 samples were reassigned randomly into three groups. After that, the entire model building and evaluation, with multiple train/test iterations, is performed by PACE. This is repeated for 1000 permutations to define the distribution of errors. The results of PACE are expressed in terms of the mean of the classification error distribution (MACE) along with the 95th and 99th percentiles of this distribution. The difference between error distribution and the ACE is representative of the nonrandom nature of the results that are obtained by the various disease prediction modeling algorithms.

Results

Patient Characteristics

The clinical and demographic characteristics of the various groups are shown in Table 1. All of the patients in the stable transplant group had stable graft function at least 6 wk before and after sample collection. The time from transplantation to sample collection was significantly shorter in stable transplant patients compared with patients with AR ($P < 0.05$). None of these patients had a history of delayed graft function. Patients with AR and BKVAN had significantly higher mean serum creatinine levels ($P < 0.05$) than stable transplant patients (Table 1). Otherwise, there were no significant demographic differences between study groups (Table 1).

The histologic findings in the AR group showed acute rejection scores of IA to IIB. Also, 16 (57%) of the patients with AR had low-grade (IA) chronic rejection changes in the allograft biopsy. In the BKVAN group, 15 (71%) of the patients were considered to have changes that would be compatible with grade IA to IIA acute rejection scores, and a similar proportion also showed features of chronic rejection. The acute and chronic rejection scores for patients with AR and BKVAN are shown in Table 2. Urinary viral PCR studies showed BKV load that ranged from 30,500 copies to $>2 \times 10^{10}$ copies/ml in all of the BKVAN urine samples. HHV-6 PCR was positive in two patients in the stable transplant group and in one patient each in both AR and BKVAN groups. One patient with AR also had simultaneously positive adenovirus, HHV-6, and CMV PCR tests, whereas two patients were positive for simultaneous adenovirus and CMV. In the BKVAN group, there were two patients with positive adenovirus PCR in urine.

Characteristics of Urine Proteome Analyzed by SELDI-TOF-MS

The Ciphergen peak detection software detected a total of 158 peak clusters. Eighty of these peaks were found in IMAC30 data, and 78 were found in the CM10 data. We assayed 27 samples in triplicate in two sessions separated by 3 mo (a total of six profiles for each of the 27 samples for a total of 162 profiles). The profiles were reproducible to a very significant extent despite the relatively low protein amount (<2 μg) reacted with each ProteinChip spot and a gap of 3 mo (Figure 1).

Visual analysis of the urinary profile data was done using Ciphergen software to identify peaks that were different between the study groups. Urine samples of stable transplant patients showed peaks with significantly higher peak intensities at the m/z values of 4.755, 6.245, 6.440, 7.672, 8.012, 8.230, 9.636, 9.870, 10.067, 10.569, and 16.918 kD compared with the samples of patients with AR or BKVAN on the analyses of both chip data. Figure 2 shows an overview of the SELDI profiles of the three groups in false gel views, and Table 3 shows further details of the characteristics of some of the differentially expressed peaks. A profile with a combination of four closely clustered peaks (IMAC30) between 6.072 and 6.440 kD together with separate peaks at 9.870 and 10.569 kD was found to be relatively constant and was present in the urine samples of 25 (86%) of the stable transplant patients (Figure 2B, frames 1 and 3). This peak pattern was seen in a significantly smaller subset of 35% of the patients with AR and 24% of the patients with BKVAN. The only peak that was seen more frequently and with a higher mean peak intensity in the AR group than in the other two groups was a peak located at m/z value of 8.854 kD in the IMAC30 data set (Figure 2B, frame 2, Table 3).

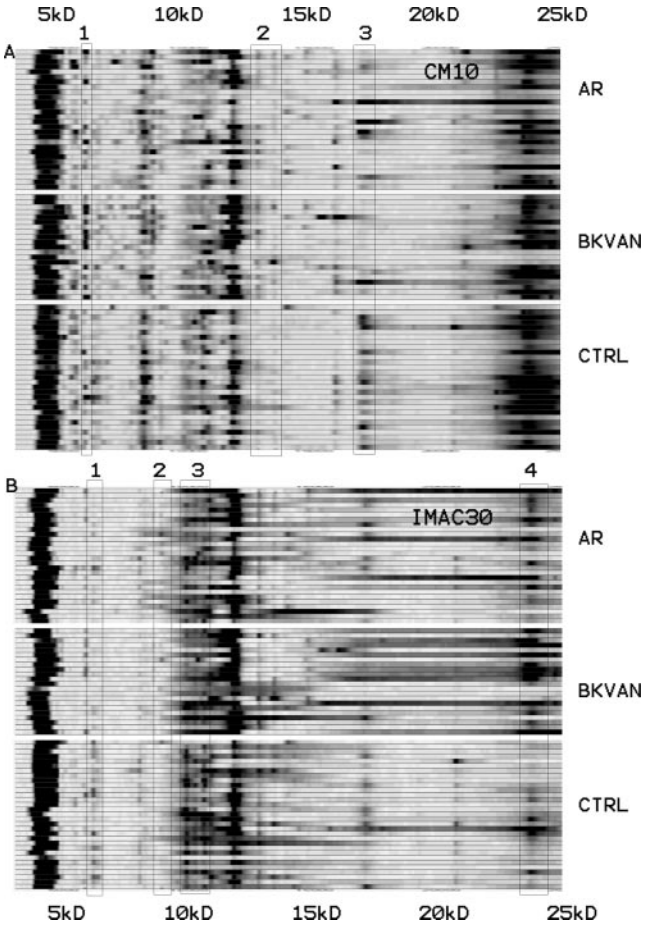

Figure 2. False-gel image of urine protein profiles corresponding to m/z 5 to 25 kD using the CM10 chip data set (A) and the IMAC30 chip data set (B). The framed m/z areas show examples of regions with significant differences between the various study groups. (A) Frame 1 = 5.872 kD (upregulated in BKVAN), frame 2 = 12.727 to 13.349 (upregulated in BKVAN), and frame 3 = 16.918 kD (upregulated in stable). (B) Frame 1 = 6.072 to 6.440 kD (upregulated in stable), frame 2 = 8.854 kD (upregulated in AR), frame 3 = 9.636 to 10.569 kD, and frame 4 = 23.482 kD (upregulated in BKVAN).

Table 3. Mean peak intensities of the most significant peaks differentiating BKVAN from other patient groups

Parameter	Normalized Mean Peak Intensity (SD)					
CM10						
m/z	5.872	11.311	11.929	12.727	13.349	23.482
BKVAN	25.8 (20.5)	2.1 (1.8)	26.5 (16.5)	15.0 (8.4)	5.8 (6.1)	11.0 (8.2)
AR	13.1 (15.0)[a]	1.3 (1.5)[a]	14.9 (13.9)[a]	7.2 (7.6)[b]	2.07 (1.4)[b]	9.1 (5.7)
stable	8.21 (11.9)[c]	1.5 (1.9)[a]	10.7 (10.3)[b]	4.5 (5.1)[c]	1.7 (0.9)[c]	13.1 (5.4)
IMAC30						
m/z	5.872	11.311	11.929	12.727	13.349	23.482
BKVAN	15.6 (11.3)	15.4 (8.1)	27.9 (13.6)	21.2 (11.4)	6.7 (5.3)	14.4 (11.9)
AR	10.9 (10.2)	8.7 (6.3)[b]	20.9 (13.7)	14.5 (12.2)	3.4 (2.6)[a]	7.5 (7.6)
stable	7.9 (8.6)[a]	8.3 (4.8)[c]	19.8 (13.4)	13.2 (11.2)[b]	3.6 (3.1)[a]	4.8 (5.5)[b]

[a] $P < 0.05$ *versus* BKVAN.
[b] $P < 0.01$ *versus* BKVAN.
[c] $P = 0.001$ *versus* BKVAN.

Univariate analyses indicated that the most significant peaks (m/z ratio [chip type]) that differentiated BKVAN samples from the other two groups were 5.872 (CM10, IMAC30), 11.311 (CM10, IMAC30), 11.929 (CM10), 12.727 (CM10), and 13.349 (CM10, IMAC30) kD peaks. All of these peaks were higher in patients with BKVAN and were seen more frequently in this group (Figures 2 and 3, Table 3). The analysis of IMAC30 data also showed significantly higher peak intensity in BKVAN *versus* stable patients for a protein with the m/z value of 23.482 kD ($P = 0.005$; Figures 2B and 3). The peak at 11.311 kD (Figure 3, panels 1 and 2) was one of the most significant peaks that differentiated between patients with BKVAN from patients with both stable graft function ($P < 0.001$) and patients with AR ($P = 0.002$).

The results of the disease prediction modeling analyses that were performed using three different models (SVM, RF, and CART) are shown in Table 4. The ACE, sensitivity, and specificity of test sets depended on the chip type and on the classification model used. The best performance characteristics with the least classification error (16.6%), maximum sensitivity (79.4%), and maximum specificity (86.5%) for differentiating BKVAN from AR group was obtained by SVM

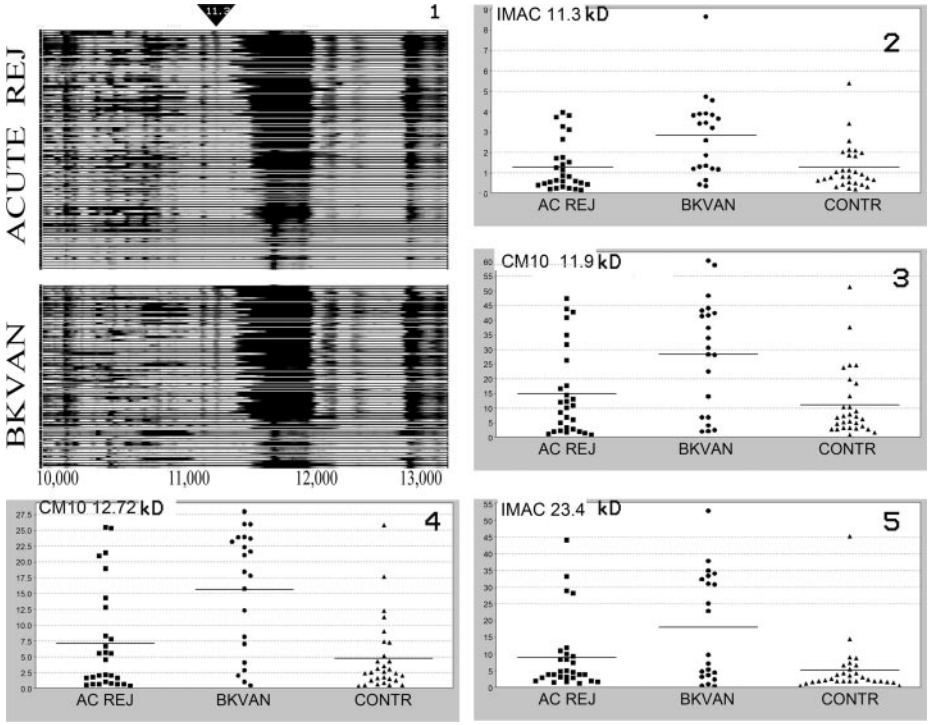

Figure 3. (1) False-gel image of 11.311-kD peak showing significant upregulation in BKVAN *versus* AR. (2 through 5) Scatter plots of the various significant classifiers in the two protein chips that differentiated BKVAN from AR and controls (IMAC30 11.3 kD [2], CM10 11.9 kD [3], CM10 12.7 kD [4], and IMAC30 23.4 kD [5]).

Table 4. Performance of various prediction models optimized for IMAC30 and CM10 chips for the BKVAN *versus* AR groups, BKVAN *versus* stable transplant groups, and AR *versus* stable transplant groups[a]

Prediction Model	IMAC30 (%)			CM10 (%)		
	BKVAN *versus* AR	BKVAN *versus* Control	AR *versus* Control	BKVAN *versus* AR	BKVAN *versus* Control	AR *versus* Control
SVM + all peaks						
ACE	29.56	23.14	39.14	41.61	35.16	40.56
sensitivity	61.09	68.97	58.94	47.77	54.99	58.39
specificity	76.64	82.11	62.47	65.41	71.63	60.36
SVM + 100 *t* test						
ACE	16.83	11.33	17.45	25.94	30.98	30.37
sensitivity	79.40	83.78	81.08	65.35	48.42	68.03
specificity	86.49	92.23	84.08	80.54	76.06	71.45
RF						
ACE	27.50	16.76	22.91	35.28	34.62	37.59
sensitivity	51.75	72.70	72.01	43.25	29.32	57.02
specificity	88.80	91.04	82.41	81.34	91.20	68.34
CART						
ACE	29.00	18.00	29.00	39.00	20.00	32.00
sensitivity	62.00	90.00	67.00	42.00	67.00	56.00
specificity	76.00	77.00	75.00	74.00	90.00	81.00

[a]The support vector machine (SVM) classifier was tested on all peaks (SVM + all peaks) and the top 100 differential peaks according to the *P* value of the *t* test (SVM + 100 *t* test). The other two models include Random Forest (RF) and Classification and Regression Tree (CART). The statistics listed include Achieved (average) Classification Error (ACE), sensitivity, and specificity.

algorithm that was performed on the top 100 classifier peaks for the IMAC30 chip data set. The PACE analysis that was performed on two of the classification algorithms (SVM and RF) indicated that the actual classification error (ACE) results were much lower than the mean error (MACE) expected by chance alone. The resulting 95th and 99th percentiles of this distribution along with the MACE are shown in Table 5.

Table 5. Permutation-based validation of the classification signal using PACE methods[a]

Parameter	IMAC (%)			CM10 (%)		
	BKVAN *versus* AR	BKVAN *versus* Control	AR *versus* Control	BKVAN *versus* AR	BKVAN *versus* Control	AR *versus* Control
SVM + all peaks						
ACE	29.56	23.14	39.14	41.61	35.16	40.56
MACE	48.61	47.96	50.25	48.77	48.27	50.34
95%	45.17	45.11	47.32	45.94	45.33	47.59
99%	44.44	44.47	46.45	45.22	45.05	47.18
SVM + 100 *t* test						
ACE	16.83	11.33	17.45	25.94	30.98	30.37
MACE	48.57	48.06	50.25	48.80	48.42	50.31
95%	46.72	46.33	48.50	46.89	46.25	48.10
99%	46.11	45.59	47.45	45.67	45.60	47.41
RF						
ACE	27.50	16.76	22.91	35.28	34.62	37.59
MACE	45.23	42.88	50.70	45.58	44.10	51.01
95%	43.67	41.33	48.86	43.94	42.66	49.12
99%	42.67	40.53	47.95	42.89	41.47	48.80

[a]The statistics shown include ACE and mean classification error (MACE) for the error distribution under the random permutation of labels and its 95th and 99th percentiles of this distribution.

Discussion

We show that proteomic profiling of urine samples may offer a noninvasive way to differentiate BKVAN from other conditions as has been shown for several other diseases (8–12,28–30). All studied patients with BKVAN had significant viremia, viruria, and biopsy-proven nephropathy at the time of sample collection. The proteomic profile of the patients with BKVAN had similarities with the AR pattern. However, we were able to detect several peaks that were differentially expressed in the BKVAN group compared with both the AR and stable function groups.

Differentiation of BKVAN from AR can be challenging both at histologic and molecular levels. A recent study by Mannon *et al.* (31) showed significant similarity of transcriptional expression of molecules associated with inflammation and fibrosis between BKVAN and AR. We found a similar overlap in the proteomic profiles of BKVAN and AR. This probably is due to the similarity of the inflammatory response and leakage of inflammation related small molecular weight proteins into urine in both conditions. However, several differentially expressed proteins were identified in the urine of patients with BKVAN in our study. Our studies can be an initial but promising phase in noninvasive biomarker development for BKVAN.

The influence of viral infections on the urine proteomics has not been studied previously in a systematic manner, and no previous studies have been reported for BKVAN. However, bacterial urinary tract infection (UTI) reportedly can have a unique proteomic profile that can be different from AR (15,16). Schaub *et al.* (15) were able to differentiate urine samples (n = 5) of kidney transplant patients with UTI from both AR and stable transplant samples using SELDI. Another study also showed the proteomic profiles of five of seven UTI samples were different from control and AR samples (16). In our studies, some of the samples also were positive for CMV, HHV-6, or adenovirus. These samples did not seem to have any different or additional peaks that could be attributed to presence of the additional virus besides BKV. Because the progression of disease that is associated with BKV generally is less aggressive (32) as compared with bacterial infections, development of noninvasive monitoring methods for BKV infection may be a clinically relevant and useful approach.

One of the advantages of SELDI is that it is inexpensive and is suitable for screening a large number of samples. However, this technique has some perceived inherent disadvantages. One of the major issues with SELDI is its relative lack of reproducibility, at least in different centers. However, in our studies, we found excellent reproducibility of the proteomic profiles when the experiments were repeated on approximately 30% of the samples after 3 mo. The reasons underlying the variable results of different centers may be due to different sample-processing protocols or approaches to the data analysis. Another disadvantage of SELDI-MS-TOF has been that it does not offer easy identification of the biomarker candidates. Therefore, studies with larger numbers of samples followed by peptide identification and immunologic assay development may be warranted.

Although no previous studies on BKVAN urinary proteomics are available, there are several reports now on urinary proteomic profiling to differentiate patients with AR from stable patients, as summarized in Table 6 (13–17). Schaub *et al.* (33) also recently showed that cleaved β2-microglobulin (11.731 kD) may be a potential marker for tubular injury in AR. The cleavage products were seen in urine proteome as three separate peak clusters (5.270 to 5.550, 7.050 to 7.360, and 10.530 to 11.100 kD). However, in other studies, including ours, the most significant peaks or peak clusters that differentiated patients with AR from stable transplant patients were different from these peaks (13,14,16,17). We found some similarities in the proteomic profile of our stable transplant patients and patients with AR compared with some of the other studies as shown in Table 6. The reasons underlying the different results of these studies probably are multifactorial. Urine is a highly complex mixture of various solutes, and its composition is influenced by diet, hydration, smoking, medication *etc.* We aimed to study the different clinical syndromes using urine samples that typically would be obtained in a standard clinical setting. We did not consider dietary factors or medications that are difficult to control. Besides these clinical variables, protein chip type and normalization approaches can be other potential confounding factors. In our study, the urine samples were normalized by loading an equal amount of protein. This same method has been used in some other studies (10,17).

The statistical analyses, including univariate and multivariate testing, of our data have shown intriguing results. Univariate analysis evaluates the ability of peaks to discriminate between two conditions, and several such peaks were identified. However, the limitation of this analysis is that peaks are analyzed in isolation. A much better discriminative model possibly can be constructed if several peaks are analyzed together. Therefore, to analyze the multivariate discriminative potential of various peaks, we evaluated various statistical prediction models: SVM, CART, and RF (22–24). In addition, the SVM model was combined with two feature selection strategies that aimed to prefilter peaks before classifiers were learned, thereby reducing potential threat of overfit. Using this approach, we found that SVM modeling of the top 100 peaks provided the best discrimination between BKVAN and AR using the IMAC30 data set. However these results come with a caveat, because one potential weakness of our study is that the sample size is not large. The various disease-prediction modeling algorithms may have limited utility in analyses of a data set of our sample size and also of others published before this study (10–12,14). Given these limitations, our analyses of SELDI profile data can be interpreted only to suggest that there is a diagnostic signal for BKVAN *versus* AR or controls in the SELDI analyses performed even on the limited sample size of our study. Our interpretation is reinforced further by the nonparametric PACE analysis (27), which also suggested that the potential diagnostic signal that was observed in patient data is significantly different from randomly reassigned data because of the marked difference of the ACE from the MACE and the 99th percentiles of error distribution curves. However, all of our analyses were based on a limited sample size, and our results on the sensitivity and the specificity of the various algorithms should be interpreted with caution. A true assess-

Table 6. Summary of the recent studies reporting proteomics biomarker candidates differentiating patients with acute AR and stable graft function and patients with various types of cancer from healthy individuals[a]

Author (Reference)	Method (Chip)/Sample	Top Classifiers (kD) in AR or Type of Cancer	Sensitivity (%)	Specificity (%)
Proteomics in acute rejection				
Clarke *et al.* (13)	SELDI (IMAC-3, H4) urine	6.5, 6.6, 6.7, 7.1, 13.4	82	100
O'Riordan *et al.* (14)	SELDI (H50, Q10, CM10, IMAC30) urine	2.003, 2.8036, 4.7563, 5.8724, 6.9906, 19.0188, 25.6657	90.5 to 91.3	77.2 to 83.3
Schaub *et al.* (15)	SELDI (NP20) urine	5.270 to 5.550, 7.050 to 7.360, 10.530 to 11.100		
Wittke *et al.* (16)	CE-MS urine	1.0276, 1.0316, 1.1686, 1.6979, 1.7078, 1.8110, 1.8138, 2.0789, 2.1211, 2.8159, 2.8389, 3.2402, 3.3596, 3.4821, 3.5161, 8.0523		
Jahnukainen *et al.* (this study)	SELDI (CM10, IMAC30) urine	4.755, 6.245, 6.440, 7.672, 8.012, 8.230, 9.636, 9.870, 10.067, 10.569, 16.918	56 to 78	63 to 81
Proteomics in cancer				
Vlahou *et al.* (11)	SELDI (SAX2)/urine	Bladder cancer	87	66
Rogers *et al.* (10)	SELDI (WCX2)/urine	Renal cancer	75	60 to 81.8
Vlahou *et al.* (12)	SELDI (SAX2, IMAC)/urine	Ovarian cancer	84.6	80

[a]SELDI, surface-enhanced laser desorption/ionization.

ment of sensitivity and specificity of the SELDI technique and the various models tested in this report cannot be determined until an independent validation set that is derived from another set of patients is assessed. Our results, therefore, may provide the basis for development of noninvasive marker tests for BKVAN if further validation studies on independent data sets could confirm our observations.

Conclusion

Proteomic marker(s) profiles, together with plasma and urine BKV PCR and clinical information, may help in making differentiation of BKVAN from AR in a noninvasive manner. Histologic verification of BKVAN probably will continue to be required for the foreseeable future, but it is likely that proteomic biomarkers could be used in deciding when a biopsy is necessary. Further studies on a larger number of patients are needed to validate our findings and to detect the identity of the significantly different peaks to develop robust, noninvasive methods for BKVAN diagnostics.

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