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PREDICTION OF RENAL ALLOGRAFT REJECTION BY URINARY PROTEIN ANALYSIS USING ProteinChip ARRAYS (SURFACE-ENHANCED LASER DESORPTION/IONIZATION TIME-OF-FLIGHT MASS SPECTROMETRY)

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ABSTRACT

Objectives. To develop a noninvasive method for the detection of renal transplant rejection using ProteinChip Arrays (surface-enhanced laser desorption/ionization time-of-flight mass spectrometry).

Methods. A total of 23 urine samples were collected from 13 patients showing biopsy-proven renal allograft rejection and from 10 patients without histologic signs of rejection. All 23 patients had clinical symptoms and signs of acute allograft rejection and underwent renal biopsy. Samples were centrifuged, and supernatants were directly spotted onto the ProteinChip arrays with different chromatographic surfaces. The obtained spectra in a range from 2 to 200 kDa were subjected to bioinformatic analysis using the method of Fuzzy c-means, followed by the establishment of rule bases and evaluation using the relevance index according to Kiendl.

Results. Several protein peaks were identified allowing differentiation between rejection and no rejection. Using two different ProteinChip surfaces, we found two biomarkers at 25.71 kDa and 28.13 kDa that gave a diagnostic sensitivity of 90% and 93% and a specificity of 80% (SAX2) and 85% (CM10), respectively.

Conclusions. Surface-enhanced laser desorption/ionization time-of-flight mass spectrometry appears to be a promising new diagnostic tool for distinguishing renal transplant patients with no rejection from those with acute rejection. UROLOGY **67:** 472–475, 2006.

Chronic allograft nephropathy due to acute rejection continues to be the chief cause of cadaver kidney transplant failure, resulting in an overall 10-year graft survival rate of approximately 50%.[1] Other known causes are preservation injury, calcineurin toxicity, and chronic rejection.

Rejection is defined as an immunologic reaction between the recipient and graft causing pronounced damage to the physiologic function of the latter.[2] Although profound knowledge of rejection etiology is still lacking, treatment can be offered through the use of corticosteroids. However, treatment success relies on early and reliable diagnosis, and clinical manifestations such as elevated creatinine, increased graft volume, and altered resistance indexes on Doppler ultrasonography are not specific. At present, the diagnosis of acute rejection can only be made by renal biopsy, which is costly, invasive, inconvenient, and carries a small risk of complications such as pain, hematuria, hematoma, arteriovenous fistula, sepsis, and shock. Furthermore, it may not detect early changes because renal function does not always correlate with the histologic alterations. Sampling error is another known problem.

This clinical situation explains the need for a noninvasive diagnostic tool that can be used to monitor the immune response to the allograft, ideally even before graft dysfunction occurs.

Recently, interest has been increasing in exploring the proteome of human urine.[3] Knowledge of the presence and amount of urinary proteins in healthy individuals may not only help to detect

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changes easily in patients but may also improve our knowledge of kidney physiology. Classic approaches to protein analysis have relied on immuno-affinity, liquid chromatography, or electrophoretic separation. Although known and applied for many years, they have a number of limitations, including a low analytic speed and limited sensitivity and flexibility.

A promising proteomic technique for the discovery of biomarkers is surface-enhanced laser desorption/ionization (SELDI) mass spectrometry-based ProteinChip technology.[4,5] First described by Hutchens and Yip,[6] the technology makes use of affinity surfaces to retain proteins based on their physicochemical characteristics, followed by direct analysis by time-of-flight mass spectrometry. Therefore, proteins retained on chromatographic surfaces can be easily purified of contaminants such as buffer salts or detergents, eliminating the need for preseparation, as required by other mass spectrometry techniques. Furthermore, the low sample requirements of this technique are ideal for small biopsies, microdissected tissue, and body fluids with low protein concentrations.[7–10]

SELDI mass spectrometry also offers a unique platform for high throughput urine protein profiling.[11] Thus, it was possible to define specific protein patterns in the urine samples of patients with bladder cancer.[12,13]

MATERIAL AND METHODS

Study Design and Samples

We conducted a retrospective study of midstream urine samples from 23 consecutive transplant patients that were subjected to SELDI time-of-flight mass spectrometry in an attempt to identify biomarkers for rejection. All were patients who had clinical rejection and showed a pronounced creatinine rise and often had clinical symptoms of rejection (eg, decreased diuresis, increased graft volume, and altered resistance indexes on Doppler ultrasonography, proteinuria, edema). All 23 patients underwent renal transplant biopsies performed using a Biopty gun with an 18-gauge needle. The renal biopsy and urine midstream collection were done within 24 hours (mostly on the same day). In this study, we did not collect and analyze urine samples taken days or weeks before or after the renal biopsy. Rejection was diagnosed by renal histopathologic analysis. All patients provided informed consent.

The renal transplant age at the biopsy/urine sampling ranged from 9 to 5466 days in both groups (rejection, n = 13: range 9 to 996 days, mean 251, standard deviation 364; no rejection: range 21 to 5466 days, mean 2105, standard deviation 2149). Four patients had delayed graft function. All four (three with rejection and one with no rejection) underwent steroid pulses after biopsy-proven rejection 22, 20, 12, and 21 days before their second biopsy/urine sampling for SELDI analysis. The creatinine levels at biopsy/urine sampling ranged from 150 to 694 μmol/L in both groups. The mean creatinine levels rose from 210.7 $\pm$ 190 μmol/L to 313.9 $\pm$ 203.9 μmol/L within 30.1 $\pm$ 24.9 days in the rejection group and increased from 210.2 $\pm$ 118.1 μmol/L to 268.1 $\pm$ 130.5 μmol/L within 78.8 $\pm$ 81.8 days in the nonrejection group (calculations included all except 4 patients with delayed graft function).

According to the reading pathologists, using the Banff-classification (1997), 4 patients had 1a, grade 1; 3 had 1a; 3 had 1b; 1 had 2a; 1 had 2b; and 1 had 2b, grade 1 in the rejection group. All patients were taking immunosuppressive medication (eg, tacrolimus, sirolimus, mycophenolate, steroids, cyclosporine, azathioprine). In the rejection group (n = 13), 4 were taking tacrolimus, sirolimus, and mycophenolate; 5 were taking cyclosporine, mycophenolate, and steroids; 2 were taking mycophenolate and steroids; 2 were taking cyclosporine and mycophenolate. In the nonrejection group (n = 10), 3 were taking tacrolimus, sirolimus, and mycophenolate; 2 were taking cyclosporine only; 1 was taking cyclosporine, sirolimus, and steroids; 1 was taking cyclosporine, mycophenolate, and steroids; 1 was taking tacrolimus and steroids; 1 was taking tacrolimus and mycophenolate; and 1 was taking azathioprine and steroids.

Urine Processing

The urine specimens were centrifuged for 30 minutes at 14,000 revolutions per minute. The supernatants were immediately frozen at −80°C.

ProteinChip Array Preparation and Analysis

The supernatant from the urine samples were analyzed on a strong anionic exchanger chip and a weak cation exchanger array (SAX2, CM10, Ciphergen Biosystems, Fremont, Calif). In brief, a 3-μL sample was spiked on, and the ProteinChip Arrays were incubated and washed with buffer and water. After application of matrix, mass analysis was performed in a ProteinChip Reader (PBS-II, Ciphergen Biosystems) according to an automated data collection protocol. Normalization of all spectra was performed using total ion current. Cluster analysis of the detected signals was done using the Biomarker Wizard Program, version 3.1 (Ciphergen Biosystems). For additional calculation, the data were exported to an Excel data sheet. The ProteinChip Array preparation and analysis took 3 to 4 hours.

Bioinformatic Analysis of ProteinChip Array Data

The resulting protein profiles were subjected to a cluster-and-rule-based data mining algorithm. The data analysis process consists of a clustering step, a rule extraction and rating step, and a rule base construction step. All steps, except for the clustering step, are controlled with respect to the given sample classification (rejection versus no rejection). The data were log-2-transformed before the normalization procedure and then normalized to the median. The preprocessed protein expressions were then grouped into two clusters—"low expression" and "high expression"—for each peak using a modified Fuzzy c-means algorithm.[14] Samples with protein expression less than the lower cluster center were assigned to the "low expression" cluster, and samples with a protein expression greater than the higher cluster center were assigned to the "high expression" cluster. The main objective of the next step—the rule extraction and rating step—was to find those features (peaks) that had the most similar membership distribution compared with the given sample class distribution. For this purpose, so-called rules were generated and rated by a statistically based rule rating measure introduced by Kiendl and Krabs.[15] Such rules can be written in the form: "IF sample belongs to cluster 'peak X high expressed' THEN sample belongs to class 'CTCL'." Rules with a rule rating measure greater than 0 were then ranked in a rule list.

A small subset of rules out of the rule list can form a rule base that can be used for automatic classification of new patient samples. A rule base contains at least one rule for every possible classification outcome. To classify a new patient sample, the cluster memberships (condition part of the rules) of all

rules out of the rule base that point to the same classification outcome (conclusion part of the rules) are added, and the sample is assigned to the class with the highest sum. To construct a rule base that gave a good representation of the data set investigated, all combinations of rules out of the rule lists were permuted, and the rule base with the smallest classification error and the smallest number of rules was chosen. The rules contained in the chosen rule base can be considered to represent markers that can distinguish between the sample classes under investigation.

RESULTS

Urine samples from 13 patients displaying biopsy-proven renal allograft rejection and 10 patients showing no rejection on histologic examination were applied to two different chromatographic surfaces. ProteinChip Array analysis resulted in measurable spectra for bioinformatic processing.

By applying cluster-and-rule-based data mining methods, two biomarkers at 25,708 kDa and 28,133 kDa were identified. Two protein chip surfaces were necessary (SAX2, CM10) and resulted in a diagnostic sensitivity of 90% and 92% and a specificity of 80% and 85%, respectively.

COMMENT

At present, renal biopsy remains an essential part of diagnosing renal allograft rejection. However, clinical treatment of patients developing graft rejection would be improved by applying noninvasive diagnostic tools, such as urinary proteins, to monitor renal transplant function. However, the several techniques to identify and compare urinary proteins, such as two-dimensional gel electrophoresis and lipid chromatography, are of limited diagnostic value.

On the basis of the hypothesis that specific changes in the urinary protein pattern could possibly be used to identify precursors and the pathologic states of renal allograft rejection, we tried to determine whether such novel proteins can be detected with mass spectrometry.

By applying cluster-and-rule-based data mining methods, two biomarkers at 25,708 kDa and 28,133 kDa were identified. In the 23 patients investigated, only two protein chips were necessary and gave a diagnostic sensitivity of 90% and 92% and specificity of 80% and 85%. Additionally, in patients 1, 19, and 21 (falsely negative for one marker), only "slight" or "focal" signs of chronic interstitial rejection were seen on histologic examination. This finding indirectly emphasizes the diagnostic value of this new method.

Moreover, using free databases (www.expasy.ch), we found possible enzymes at the molecular weight of 25,708 kDa and 28,133 kDa that might be involved in the pathologic states of renal allograft rejection. One possible candidate is glutathione-S-transferase (25,708 kDa). The main function of enzymes belonging to the glutathione-S-transferase family is to protect cells against the potential toxicity of lipid hydroperoxides generated during oxidative stress. Patients with chronic renal failure and those who have undergone renal transplantation show decreased glutathione-S-transferase levels as an indicator of oxidative stress.[16,17] Tissue plasminogen activator appears to be another possible protein (28,133 kDa) that exhibits increased activity after transplantation in serum[18] and urine.[19]

Two other investigators have performed urinary protein profiling to correlate different clinical outcomes after renal allograft transplantation. Both investigators found protein peaks that were different from those we presented. Clarke *et al.*[13] reported on two rejection biomarkers at 3.4 and 10.0 kDa in 17 renal transplant patients, and Schaub *et al.*[3] found three peak clusters between 5 and 11 kDa in 18 renal transplant patients. These differences may have been related to the different protein chips used and the lack of a standardized procedure for the use of the SELDI method.[20] Schaub *et al.*[11] investigated factors that may influence reproducibility and peak detection. Matrix composition, instrument settings, the use of midstream or first-voided urine, and urine contamination (microscopic hematuria), as well as urine dilution had a substantial impact on urinary protein profiling.[11] They also did not use bioinformatics to assign protein peaks to a specific clinical outcome but included control populations such as patients with acute tubular necrosis, recurrent or de novo glomerulonephritis, urinary tract infection, and cytomegalovirus. Clearly, this appears to be the best scientific approach to distinguish rejection from its differential diagnosis. However, for clinical management, we need biomarkers that help diagnose "real" cases of acute rejection within the group of patients in whom rejection is clinically suspected. All 23 patients of this study displayed symptoms and signs of acute allograft rejection and therefore underwent renal biopsy. Of the 23 patients, 13 had typical histologic signs of rejection.

There is no doubt that protein isolation and identification will be necessary not only to understand basic rejection pathologic findings, but also to monitor renal graft function noninvasively using urinary protein analysis in the future.

CONCLUSIONS

Our investigation has provided evidence that using protein analysis could be a viable noninvasive technique for the diagnosis of acute allograft rejection that could, in contrast to renal biopsy, be repeated at any time to observe renal allograft function. However, more samples must be analyzed to verify our results and other differential protein

markers should be identified and their function deciphered. Finally, additional research and analysis of urinary proteins could eventually lead to the detection of key proteins involved in pathologic states after renal allograft transplantation.

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