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Prediction of Acute Cellular Renal Allograft Rejection by Urinary Metabolomics Using MALDI-FTMS

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Abstract: The present study investigated small molecule analysis of urinary samples as a noninvasive method to detect acute cellular renal allograft rejection. Matrix-assisted laser desorption/ionization Fourier transform mass spectrometry (MALDI-FTMS) was used to analyze 15 urinary samples from transplant patients with different grades of biopsy showing improved clinical acute cellular rejection (ACR) and 24 urinary samples from 8 transplant patients without evidence of rejection. Seven small molecules demonstrated highly successful diagnostic performance (m/z): 278.1 ($t = 3.398$, $p = 0.004$), 293.0 ($t = 2.169$, $p = 0.048$), 294.1 ($t = 2.154$, $p = 0.05$), 382.2 ($t = 2.961$, $p = 0.010$), 383.3 ($t = 2.270$, $p = 0.040$), 402.2 ($t = 2.994$, $p = 0.010$), 424.0 ($t = 2.644$, $p = 0.019$). Kidney transplant patients with ACR could be distinguished from those without ACR using four individual small molecules with a specificity of 100%. In conclusion, the combination of MALDI-FTMS technology with a clear definition of patient groups can detect urine small molecule associated with ACR.

Keywords: MALDI-FTMS • acute cellular rejection • metabolomics • urine • kidney transplantation

Introduction

Kidney transplantation is an effective therapy to improve the survival and quality of life for patients with end-stage renal disease (ESRD). Allograft failure is still one of the most common causes for ESRD,[1] although both short-term and long-term survival of renal allograft have been improved over the last 15 years.[2] Acute rejection (AR) remains to be the major impediment to the success in kidney transplantation. It would be more successful to treat those patients, if AR could be diagnosed earlier to avoid to the develolment of graft loss either immediately or as a result of chronic allograft nephropathy.[2,3]

Successful management of AR requires early detection and adequate treatment. The monitoring of renal function, however, is still dependent on tranditinal technologies, for example, serum creatinine levels, total urine output, body temperature, blood pressure or blood glucose. Available diagnostic methods include clinical presentation, biochemical parameters, and renal allograft biopsy. These often are nonspecific and subjective based on the clinical experience of the transplant physician. For example, elevated serum levels of creatinine, usually as the first available indication of allograft dysfunction, is not really AR-sensitive or specific.[4] It might not reflect the early changes, due to the lack of correlation between renal function and histologic improvement.[5–7] Thirty percent of rejection evidenced by biopsies was considered as a stable renal function or successful treatment.[6–9]

Percutaneous renal biopsy has its own limits for routine application, for example, high cost, invasiveness, inconvenience, risk of complications. sampling errors and subsequent disparities,[10–13] although it is regarded as the 'golden standard' for the diagnosis of rejection and delayed graft function. Systemic physiology and pathophysiology have been suggested to be reflected by polypeptides present in body fluids such as serum or urine.[14] Small molecule metabolites may be useful to discover biomarkers for monitoring kidney function and AR, since the kidney is fundamentally a metabolic organ. Understanding protein profiles in the urine not only improves our knowledge of renal graft physiology,[15,16] but also allows to identify novel proteins associated with pathologic states. The aim of the present study is to search metabolite levels of biomarkers in urine which are more detectable and reflective of kidney function, more AR-specific and correlative to clinical findings from biopsies. Metabolites are the end products of cellular regulatory processes, regarded as the ultimate response of biological systems to challenges and as an efficient application for diagnosis.[17,18] In the present study, we applied matrix-assisted laser desorption/ionization Fourier transform mass spectrometry (MALDI-FTMS) as a rapid profile of a biological sample and an appropriate chemometric method, Principle Component Analysis (PCA), as a data evaluation tool to investigate potential metabolic biomarkers reflecting AR. We combined MALDI-FTMS and patient selection criteria, including allograft histology, function and clinical fingdings to monitor the small molecules, varied between 100 and 1000 Da, in the urine of the transplant patients to detect small molecules associated with acute cellular rejection (ACR).

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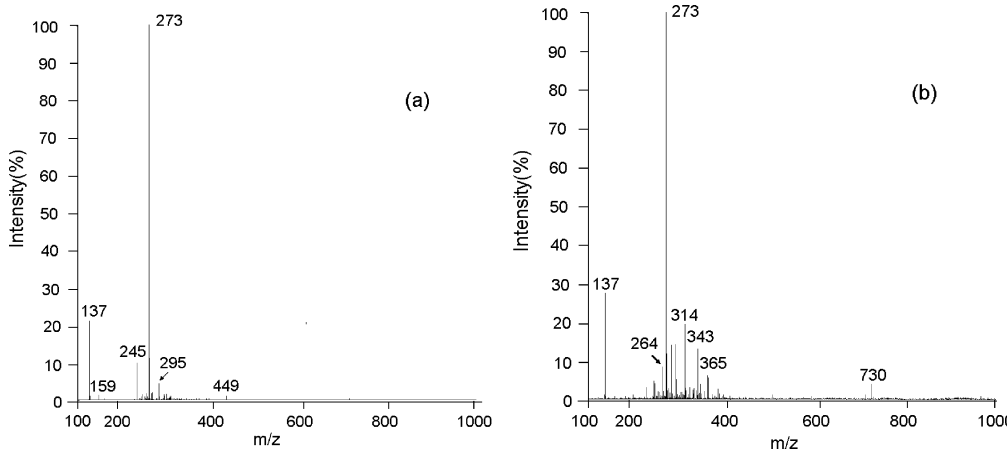

Figure 1. Mass spectra from (a) blank background (DHB matrix) and (b) a urine sample.

Materials and Methods

Materials. Methanol in HPLC grade and MilliQ water (Millipore, ELPaso, TX) were used. 2, 5-Dihydroxy benzoic acid (DHB; Sigma) was saturated in 50% ethanol. Trifluoroacetic acid (TFA, ≥99.5%) was from Fluka.

Patients and Sample Collection. Fifteen urine samples were collected from 5 transplant recipients (every patient with 3 medistream urine samples at different times) with biopsy showing improved ACR (Banff 97′ Ia to IIb) before the treatment of the rejection, and 24 urine samples from 8 patients (every patient with 3 medistream urine samples at different times) with no rejection at Zhongshan Hospital (Shanghai, China). Informed consents were obtained from the patients. All cases of ACR were confirmed by biopsy specimens evaluated by an independent, blinded pathologist, while patients with non-ACR were followed-up for more than 6-month with stable renal function. Each of the specimens was collected in two 10-mL sterile polypropylene centrifuge tubes, one for the following analysis and another for comparison of doubtful samples. Aliquots of urine specimens were taken with a disposable 1.5-mL low-absorption tube, and frozen at −80 °C until further analysis.

Preparation of Urine Samples and MALDI-FTMS Profile. For evaluation, urine samples were thawed and centrifuged for 30 min at 10 000 rpm to remove the cells and fragments. A total of 0.5 mL of the supernatant was transferred to a new tube and mixed with matrix. A 1:5 mixture of the sample and a solution of saturated DHB in CH_3OH/water/TFA (50:50:0.1) was applied to the MALDI target. The target was inserted into the ion source after drying. The experiments were performed on a FTMS (Ionspec, Irvine, CA) equipped with a 4.7 T actively shielded superconducting electromagnet (Cryomagnetics, Oak Ridge, TN). The external Ionspec MALDI ion source has an air-cooled ND:YAG laser (355 nm, New Wave, Fremont, CA) with a gradient filter for adjusting the UV-laser power. Every sample was analyzed at least three times and the mean data was adopted.

Statistics. Mass spectra generated by MALDI-FTMS were plotted using IonSpec Fourier Transform Mass Spectrometer Data System. DHB background data were removed and data of m/z and relative abundance were collected and examined visually to select small molecules as biomarker candidates for transplant patients with AR. The relative abundance of selected small molecules was subjected to Independent-Sample t test (SPSS 11.0) to statistically identify those small molecules with the best ability to distinguish between the patient populations. A p value < 0.05 (two-tailed test) was considered significant. PCA program was used to deal with AR biomarker candidates. The data of m/z and relative abundance were imported into the PCA, where loading graphs were made to express the status of the urine samples.

Results

Mass spectra from kidney transplant patients in the m/z varied from 100 to 1000, revealing 10^7 distinct peaks of interest that showed difference between blank background and the urine samples. A sample of the spectra is showed in Figure 1. According to the Independent-Sample t test, the m/z of small molecules with the best ability to distinguish between ACR and non-ACR patient populations were 278.1, 293.0, 294.1, 382.2, 383.2, 402.2 and 424.0. The detailed relative abundances of these 7 small molecules were shown in Table 1.

The experiment data were subjected to Independent-Sample t test and the 7 small molecules demonstrated highly successful diagnostic performance: 278.1 (t = 3.398, p = 0.004), 293.0 (t = 2.169, p = 0.048), 294.1 (t = 2.154, p = 0.05), 382.2 (t = 2.961, p = 0.010), 383.3 (t = 2.270, p = 0.040), 402.2 (t = 2.994, p = 0.010), 424.0 (t = 2.644, p = 0.019). Four small molecules (278.1, 382.2, 383.2, 424.0) have 100% of the specificity to differ between ACR and non-ACR (Table 2).

To evaluate the ACR-specific value of these 7 small molecules, PCA program was applied to deal with the data. Figure 2 shows the disposition of the samples collected from transplant patients with or without ACR. Almost all the urine samples from the non-ACR patients were concentrated on a certain region, and from ACR patients diffusely distributed to different regions, as shown in Figure 2. It demonstrated the difference of metabolites in urine samples between patients with or without ACR.

Discussion

This study demonstrates that ACR of renal allograft could cause significant changes in the urine metabolites, which occurred even in cases with subclinical and histologically mild rejection. Specific small molecules were identified for distinguishing patients with ACR, types or degrees of the ACR. This might compensate biopsies-associated sampling errors from biopsies and subsequent disparities between

Table 1. The Detailed Relative Abundance of Small Molecules with the Best Ability of Distinguishing ACR[a]

		278.1	293	294.1	382.2	383.2	402.2	424
R-1	R-1-1	0	1.6	0	3.87	0	6	1.32
	R-1-2	0	0	1.41	8.08	0	16.29	0
	R-1-3	5.24	0	3.15	0	0	0	0
R-2	R-2-1	1.7	0	0	0	0	0	1.2
	R-2-2	3.1	1.56	0	0	0	0	1.38
	R-2-3	0	0.59	0	0	0	0	0
R-3	R-3-1	0	2.77	0	15.67	3.71	37.74	4.71
	R-3-2	0	1.79	0	6.65	1.01	9.01	0.99
	R-3-3	0	14.24	0	16.67	3.6	24.96	2.61
R-4	R-4-1	2.86	6.12	5.95	2.07	0	6.46	2.47
	R-4-2	4.16	2.28	6.05	4.72	1.57	26.71	11.28
	R-4-3	2.12	0	1.39	6.79	1.62	20.05	5.95
R-5	R-5-1	0.32	0.15	0.11	0	0	0	0
	R-5-2	2.74	0.57	0.71	0	0	0	0
	R-5-3	0.78	0.25	0.21	0	0	0	0
N-1	N-1-1	0	0	0	0	0	0	0
	N-1-2	0	0	0	0	0	0	0
	N-1-3	0	0	0	0	0	0	0
N-2	N-2-1	0	0	0	0	0	0	0
	N-2-2	0	0.51	0	0	0	0	0
	N-2-3	0	0	0	0	0	0	0
N-3	N-3-1	0	0	0.92	0	0	0	0
	N-3-2	0	0	0	0	0	0	0
	N-3-3	0	0	0	0	0	4.98	0
N-4	N-4-1	0	0	0	0	0	0	0
	N-4-2	0	0	0	0	0	0	0
	N-4-3	0	0	0	0	0	0	0
N-5	N-5-1	0	0	0	0	0	0	0
	N-5-2	0	0	0	0	0	0	0
	N-5-3	0	0	0	0	0	0	0
N-6	N-6-1	0	0.48	0	0	0	0	0
	N-6-2	0	0	0	0	0	0	0
	N-6-3	0	0	1.11	0	0	0.57	0
N-7	N-7-1	0	0	0	0	0	0	0
	N-7-2	0	0	0	0	0	0	0
	N-7-3	0	0	0	0	0	0	0
N-8	N-8-1	0	0	0	0	0	0	0
	N-8-2	0	0	0	0	0	0	0
	N-8-3	0	0	0	0	0	0	0

[a] Note: R = Acute rejection patients; N = No acute rejection patients. The Banff 97′, used to grade the rejection and the grade of the patients with acute cellular rejection, was, respectively, II A, I B, I B, I A, I A∼II A acute cellular rejection.

Table 2. The Sensitivity and Specificity of the 7 Small Molecules in Distinguishing Acute Cellular Rejection and Non-Rejection Patients

	278.1	293	294.1	382.2	383.2	402.2	424
Sensitivity	60.0%	73.3%	53.3%	53.3%	33.3%	53.3%	60.0%
Specificity	100%	84.6%	80.0%	100%	100%	80.0%	100%

clinical and microscopic findings. Our aim was to develop AR-specific, noninvasive and multiple-accessible biomarkers to monitor the patients with renal allograft, predict the prognosis after the transplantation, and enhance the surveillance of renal allografts and recipients. The present study focused more on urine sample due not only to the easiness of sampling without need of medical ethics, but also to the importance of metabolic production. However, it would be important to correlate the alterations in the urine with those in blood/serum, which should be completed in the future study.

There is an increasing interest in the development of urine biomarkers for detection of renal AR as an alternative to percutaneous needle biopsy. Urine proteome analysis with surface-enhanced laser desorption /ionization time-of-flight mass spectrometry (SELDI-TOF-MS) was evaluated in renal transplant recipients, and findings of polypeptide patterns with and without renal transplant were variable. SELDI-TOF-MS offers many advantages for protein profiling in urine, for example, less volume of samples, simple chip preparation, easier and quicker analysis, less interference with MS analysis, and optimal purification conditions for a protein. However, standardization of analysis conditions is essential, and both extrinsic and intrinsic factors must be taken into account for accurate data interpretation.[19] Previous studies demonstrated the potential for urine biomarker discovery using SELDI-TOF-MS proteomics analyses.[20–24]

In addition to SELDI-TOF-MS, capillary electrophoresis-mass spectrometry (CE-MS) was used to screen urine biomarkers for detection of renal AR.[25] There is a great variation of methodology of data analysis, philosophy of biomarker discovery and strategy of biomarker validation. It is important to note that the technology allows more advanced chip surfaces available today, small fractions of detectable pro-

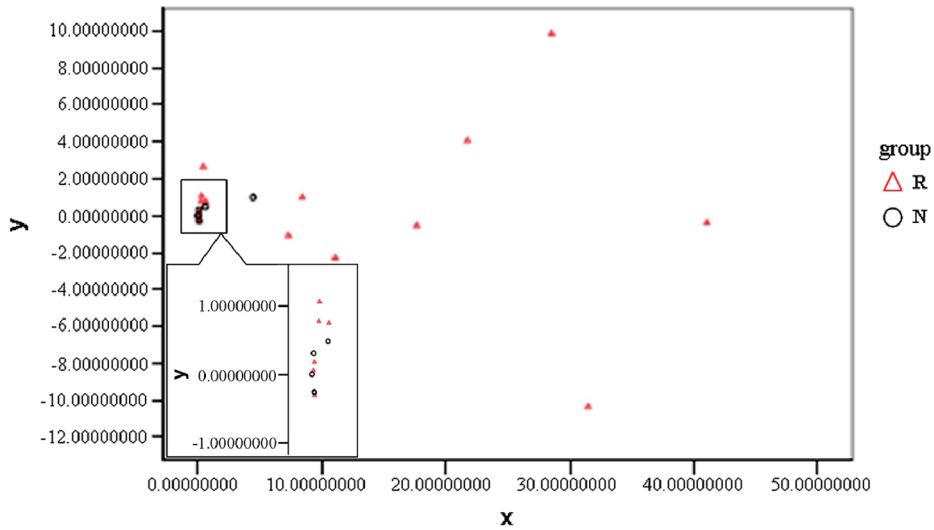

Figure 2. The loading graph of 39 specimens of the transplant patients with acuterejection and no acute rejection. R = acute rejection patients; N = no acute rejection patients.

teins, and variability of biomarkers. Variations of peak detection and data analysis can also result in different biomarker reported from the same data set. However, small molecular metabolites may be the most useful biomarkers for monitoring kidney function and detecting renal adverse events, since those metabolites and toxins are filtered in the kidney. It has been suggested that changed metabolite levels in urine could be both more detectable and reflective of kidney function than subtle changes to the renal proteome or transcriptome.[26]

In contrast to proteomic analysis of urine samples on molecular weight > 1500 Da, we used metabolomics, a high-throughput measurement of large numbers of small molecule metabolites (<1500 Da) and a part of the metabolome. The metabolome is the collection of all small molecule metabolites (endogenous or exogenous) that can be found in a cell, organ or organism. In clinical chemistry, most metabolites are typically identified and quantified using colorimetric chemical assays. In metabolomics, a large number of metabolites are measured using nonchemical, noncolorimetric methods such as gas chromatography−mass spectrometry, tandem mass spectrometry or nuclear magnetic resonance spectroscopy. In the present study, we monitored small molecules which varied from 100 to 1000 Da and especially from 200 to 600 Da. There are some merits to the analysis of small molecules in the urine using MALDI-FTMS: (1) MALDI can produce intact singly charged molecular ions in the gas phase with molecular weights up to and beyond 10^6 Da and offer a promising high-throughput and rapid tool; (2) small molecules are the metabolites of polypeptides, protein and other molecules, so they may have higher sensitivity and specificity; (3) we may presume certain whole metabolic pathways of the allograft rejection according to the small molecules that were served as rejection biomarkers.

Each metabolite has its own biological significance in the pathophysiology and pathogenesis of the disease. It is important to validate these potential biomarkers in all patients with renal transplantation, corresponding with the rejection responses, severity, duration and long-term prognosis. Early intervention with immunosuppressive drugs or other strategies may be useful in avoiding complete graft loss, if these markers could predict those that will develop allograft rejection. Furthermore, we also realize that there is a great need of validating the biomarkers selected from the present study in a large number of samples and patient populations, and of doing more patient follow-up and identification of the structure of these small molecules in our future investigations.

In conclusion, the present study described a small molecule profile of the urine from patients with renal transplantation to monitor the development of AR, using the combination of proteomics and clinical findings based on allograft histology, allograft function, and clinical course. We found the potential association between these small molecules and acute renal allograft rejection. These may act as biomarkers to monitor AR severity, duration and outcomes after renal transplantation and play an important role in identifying patients with subclinical AR. Further validation in a large population of patients with AR after transplantation is needed to optimize and minimize the allograft biopsy.

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