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Preliminary Application of WCX Magnetic Bead-Based
Matrix-Assisted Laser Desorption Ionization
Time-of-Flight Mass Spectrometry in
Analyzing the Urine of Renal Clear
Cell Carcinoma

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Objective To evaluate the application of weak cation exchange (WCX) magnetic bead-based Matrix-Assisted Laser Desorption Ionization Time-of-Flight Mass Spectrometry (MALDI-TOF MS) in detecting differentially expressed proteins in the urine of renal clear cell carcinoma (RCCC) and its value in the early diagnosis of RCCC.

Methods Eleven newly diagnosed patients (10 males and 1 female, aged 46-78, mean 63 years) of renal clear cell carcinoma by biopsy and 10 healthy volunteers (all males, aged 25-32, mean 29.7 years) were enrolled in this study. Urine samples of the RCCC patients and healthy controls were collected in the morning. Weak cation exchange (WCX) bead-based MALDI-TOF MS technique was applied in detecting differential protein peaks in the urine of RCCC. ClinProTools2.2 software was utilized to determine the characteristic proteins in the urine of RCCC patients for the predictive model of RCCC.

Results The technique identified 160 protein peaks in the urine that were different between RCCC patients and health controls; and among them, there was one peak (molecular weight of 2221.71 Da) with statistical significance ($P=0.0304$). With genetic algorithms and the support vector machine, we screened out 13 characteristic protein peaks for the predictive model.

Conclusions The application of WCX magnetic bead-based MALDI-TOF MS in detecting differentially expressed proteins in urine may have potential value for the early diagnosis of RCCC.

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RENAL clear cell carcinoma (RCCC) usually does not present obvious symptoms in its early stage. Currently, we are in short of tumor markers of RCCC to make an accurate and sensitive diagnosis, and to monitor its development in clinical practice. Most tumor markers are proteins that are abnormally expressed. By means of dynamic analysis of proteins, we can detect the minor index and early symptoms of diseases. The emphasis of proteomics is to identify the proteins that are differentially expressed in cancer. Therefore, proteomics provides an ideal technology platform for screening tumor markers of renal cell carcinoma.

Protein fingerprinting is a new protein technology developed in recent years. It can capture subtle changes in protein during the process of tumor evolution, which makes early diagnosis possible.¹ Proteomics provides a novel idea and research platform for the diagnosis of renal cell carcinoma. Tested specimens of renal cell carcinoma in proteomics technology are mainly serum, urine and cell lines. In this study we used bead-based MALDI-TOF MS to identify the characteristics of protein expression in the urine of patients with renal clear cell carcinoma, in order to find the specific protein peaks that can be used for an early diagnosis and treatment of renal cell carcinoma.^{2,3}

MATERIALS AND METHODS

Subjects investigated

This study has been approved by the institutional ethics review committee. From March 2010 to April 2010 in the Department of Urology, Peking Union Medical College Hospital, a total of 11 patients with newly diagnosed renal clear cell carcinoma were enrolled in the study, including 10 males and 1 female, aged from 46 to 78 years, with an average of 63.0 years old. Clinical diagnosis was established by imaging examinations such as ultrasonography, CT, or MRI. The pathological diagnosis of RCCC was confirmed by biopsy or surgery. Clinical TNM staging was as following: 3 cases of stage Ia, 7 cases of stage Ib, 1 case of stage II, and 1 case of stage IV. Six cases had laparoscopic nephron sparing surgery, 4 cases had laparoscopic radical nephrectomy, and 1 case had targeted drug therapy of SUTENT after biopsy. The urine routine examination is completely normal.

Ten healthy volunteers were recruited as normal controls. All were males, aged from 25 to 32, with an average of 28.7 years old. They were negative in physical examination, and the routine examinations of urine were completely normal.

Urine specimen collection and preparation

We collected the second middle clean urine of every participant at 7 o'clock in the morning after fasting from solids and liquids for 9 hours. After centrifugation at a speed of 2000 rpm for 5 minutes, the supernatant was obtained and packed in Eppendorf tubes, frozen in a low-temperature refrigerator under -80°C till use. The procedure was done within 30 minutes.

After being taken out from refrigerator, 30ul urine was put into a tube that contained magnetic bead binding buffer, magnetic beads, and magnetic beads elution buffer. They were homogeneously mixed together for at least five times, and kept at room temperature till use.

Preparation of weak cation bead

10ul bead binding buffer and 10ul beads (Bruker Daltonics Inc. USA) were mixed in a 200ul sample tube; then 5ul urine was put into the sample tube and mixed together. The sample tube was put into a magnetic bead separator, and supernatant liquid was sucked out after separation. Then added 100 ul beads in the sample tube, put into a magnetic separator, and then removed the supernatant. This procedure was repeated twice to ensure that the suspension was absorbed completely. Next, 5ul beads elution buffer was put into the sample tube, and the tube was placed in a magnetic bead separator. After the magnetic beads were fully separated from the suspension liquid, the supernatant was transferred into a 50ul sample tube. Then 5ul magnetic stabilization buffer was put into the tube and mixed for use.

Examination of standard sample

We dissolved 1ul standard sample with 10ul matrix at room temperature. 1ul mixture liquid was placed in the standard position of target Anchor chip at room temperature and in dry environment. The Anchor chip target was put into the mass spectrometer (Autoflex™, Bruker Daltonics Inc., USA), with the selection method of LP Clinprot and the acquisition range of 1000-13000Da. The composition and the mass spectrometry of the standard sample were as shown in Fig. 1.

Examination of urine samples

At room temperature in a dry environment, 1ul of sample-treated magnetic beads was placed in the sample target position of Anchor chip. American MALDI-TOF-MS mass spectrometer (Autoflex™, Bruker Daltonics Inc., USA) was applied. The Anchor chip target was placed into the mass spectrometer, with a linear mode and an acquisition range of 800-10 000 Da.

Data processing

According to the characteristics of the overall waveform, the unqualified atlas was removed, and molecular weights in the low molecular weight area were corrected. The range of molecular weight was 1 000-10 000 Da, and the molecular weight was adjusted by the standard sample. Data of mass spectrometry were analyzed following the workflow of ClinProTools2.2 (Bruker Daltonics Inc., USA), which is a mass spectrometry-based biomarker analysis software to screen the characteristic proteins in the urine of renal clear cell carcinoma for diagnosis. Genetic algorithms and the support vector machine were used to establish a predictive model. $P < 0.05$ was considered

statistically significant.

RESULTS

Difference of the protein peaks of urine between RCCC patients and normal controls

Mass spectrometry identified 160 protein peaks in the urine of patients with RCCC that were different from those of the normal controls. The molecular weight ranged from 1000 to 10 000 Da (Fig. 2). The gel simulation schematic diagram demonstrated a protein peak with the molecular weight of 2221.71 Da, which was significantly different from the normal controls ($P = 0.0304$) (Fig. 3).

Figure 1. The composition and the mass spectrum of the standard sample.

Figure 2. Protein peak spectrum of renal clear cell carcinoma patients and the normal controls.

The gray is the average spectrum of the control group, and the red is the average spectrum of the group of renal clear cell carcinoma.

Figure 3. The gel simulation schematic diagram of urine protein spectra.

The upper square is of RCCC patients, and the lower square is of normal controls.

Establishing diagnosis model with genetic algorithms

A diagnostic model for RCCC was established with genetic algorithms by ClinProTools2.2 software. Genetic algorithms identified 13 specific protein peaks for the predictive model. The molecular weights of these protein peaks were listed in the Table 1.

DISCUSSION

The incidence of renal cancer has been steadily increasing worldwide.^{4,5} The early stage of renal cell carcinoma usually has no symptoms, and the diagnosis mainly relies on medical imaging such as ultrasound, CT and/or MRI. When a patient has the "kidney cancer triad" that is hematuria, lumbago and abdominal mass, it is often in the advanced stage. It is urgent to find accurate and sensitive markers for screening and the early diagnosis of renal cell carcinoma in order to improve the outcome of this life-threatening disease.

MALDI-TOF MS technology was developed on the base of traditional 2D gel electrophoresis proteomics technology. It collects a variety of protein peaks by molecular weight ranged from 1 000 Da to 10 000 Da. The molecular weights of many identified significant proteins are below 5 000 Da, while the minimum molecular weight that classical proteomics technique, such as 2D gel electrophoresis, is capable to detect

is between 4 000 Da and 5 000 Da. Therefore, MALDI-TOF MS technology has obvious advantages in identifying differentially expressed proteins of small molecular weight. It is simple, rapid, with high purity, and suitable for clinical application.⁶⁻⁸ When the magnetic beads chip is adopted, protein isolation can be repeatedly implemented for times,⁹ which results in good consistency, feasibility and credibility.

In this study we used ClinProTools software to screen urinary proteins of RCCC patients in order to identify the featured proteins that were different from those of normal controls. Mass spectrometry identified 160 protein peaks, with molecular weights ranged from 1 000-10 000 Da. Among them we found a significant protein peak with the molecular weight of 2 221.71 Da, which can be used as a potential marker for the early diagnosis and follow-up of renal clear cell carcinoma.

Genetic algorithms were used in this study to establish a predictive model based on the characteristic protein spectrum we found in RCCC patients. A genetic algorithm is a global optimization algorithm, which has been developed in recent years. The ClinProTools2.2 software we used in this study comprehensively integrated biological genetics, natural selection, heredity, variation and other mechanisms to achieve individual adaptability and improve problem solving effect.¹⁰ With this technique support, this study established a predictive model of thirteen characteristic protein peaks in urine for the diagnosis of RCCC. Further investigation with an expanded sample is needed to confirm the characteristic expression of proteins found in this study, and the validity of the predictive model needs to be tested with fresh samples in clinical practice.

In conclusion, this preliminary study of proteomics technology identified characteristic protein peaks in the urine of patients with renal clear cell carcinoma, and established a predictive model without blind validation. The results of this study implied that applying proteomics technology to test urine is promising for screening suspicious patients of RCCC, and further investigations are needed in the future.

Conflict of interests statement

The authors have no conflict of interests to declare.

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Table 1. The selected 13 specific protein peaks of urine for the diagnostic model of RCCC ($n=11$)

Index	Molecular mass (Da)	Start mass (Da)	End mass (Da)	Weight in diagnostic model
45	2544.23	2539.39	2547.26	1.48
46	2549.69	2547.26	2554.68	1.11
77	3675.59	3665.07	3680.1	1.31
85	4010.08	4004.66	4020.37	0.24
92	4270.51	4262.22	4280.24	1.23
98	440.75	4417.21	4431.27	0.60
102	4820.53	4812.39	4830.27	0.89
128	6635.97	6621.02	6643.5	1.04
137	8015.2	8007.3	8022.17	0.10
147	8659.21	8646.72	8666.43	1.33
150	9013.4	9005.82	9019.82	0.83
154	9198.44	9190.34	9204.48	0.31
160	9842.68	9833.65	9825.85	0.33

RCCC, renal clear cell carcinoma.

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ERRATUM

In the Volume 32 Number 2 of Chinese Medical Sciences Journal, in the original article titled *Prevalence of Carbapenem-Resistant Klebsiella Pneumoniae (CRKP) and the Distribution of class 1 Integron in Their Strains Isolated from a Hospital in Central China*, the author Chunfang Yu and the author Bei Li contributed equally and should be both marked as the corresponding author. The correspondence information of the author Chunfang Yu is: Tel: 86-0719-8891151, 86-15972591221; E-mail: 3044699970@qq.com