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Review

# Biomarker discovery for kidney diseases by mass spectrometry

Toshimitsu Niwa *

*Nagoya University Hospital, Department of Clinical Preventive Medicine, 65 Tsurumai-cho, Showa-ku, Nagoya 466-8560, Japan*

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**Abstract**

By the development of soft ionization such as matrix-assisted laser desorption/ionization (MALDI) and electrospray ionization (ESI), mass spectrometry (MS) has become an indispensable technique to analyze proteins. The combination of protein separation and identification such as two-dimensional gel electrophoresis and MS, surface-enhanced laser desorption/ionization-MS, liquid chromatography/MS, and capillary electrophoresis/MS has been successfully applied for proteome analysis of urine and plasma to discover biomarkers of kidney diseases. Some urinary proteins and their proteolytic fragments have been identified as biomarker candidates for kidney diseases. This article reviews recent advances in the application of proteomics using MS to discover biomarkers for kidney diseases.


*Keywords:* Mass spectrometry; Biomarker; Kidney disease; Proteome

## Contents



* Tel.: +81 52 744 1980; fax: +81 52 744 1954.
*E-mail address:* tniwa@med.nagoya-u.ac.jp.

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## 1. Introduction

Kidney diseases such as diabetic nephropathy and IgA nephropathy are associated with proteinuria that is caused by increased glomerular permeability. As treatment is disease specific, it requires knowledge of the underlying process. A renal biopsy is required to make a definitive diagnosis of the cause of the disease. Some patients are not suitable or are at higher risk for a biopsy due to several factors such as bleeding disorders, obesity, severe hypertension and end stage renal disease. Urine testing for biomarkers could replace renal biopsy as a simple, safe, and accurate test that could be repeated to follow the progression of the disease and to monitor the response to therapy. Research of biomarker discovery on the basis of proteome analysis of urine has recently advanced [1–5].

Proteomics employs a protein separation method such as two-dimensional gel electrophoresis (2DE) and the identification of proteins using mass spectrometry (MS). One of the objectives of clinical proteomics is the identification of biological markers of disease. To accomplish this, it is necessary to have a normal proteome of urine. Comparison of the normal urinary proteome with the urinary proteome from patients with a defined disease can detect proteins expressed differentially from one another [4]. This article reviews recent advances in the application of proteomics using MS to discover biomarkers for the diagnosis of kidney diseases.

## 2. Separation and identification of proteins

A combination of a protein separation technique and MS has been used to discover biomarkers for kidney diseases. Protein separation techniques include two-dimensional gel electrophoresis (2DE), high-performance liquid chromatography (HPLC), capillary electrophoresis (CE), and multi-dimensional methods such as HPLC–HPLC, HPLC–CE.

There are two strategies to discover protein biomarkers, so-called top–down and bottom–up. In the top–down strategy, the proteins are first separated by 2DE or the other separation methods, and the purified proteins are digested by trypsin, followed by MS analysis of the tryptic peptides. In the bottom–up strategy, also called the shotgun method, proteins are first digested by trypsin into peptides, and the resulting peptides are subsequently separated by strong cation exchange chromatography coupled with reverse-phase chromatography, and identified by MS.

### 2.1. Two-dimensional gel electrophoresis (2DE)

O'Farrell [6] first reported a 2DE technique that separates proteins (>10 kDa) according to two intrinsic characteristics: isoelectric point and molecular mass. Separation is accomplished in two steps; proteins are first separated by electrofocusing (proteins migrate to their isoelectric point in a pH gradient) and then in a perpendicular dimension by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) (proteins migrate on the basis of their molecular mass). 2DE is a high-resolution separation technique that can be coupled with protein identification by MS. 2DE can visualize differences in post-translational modifications when these changes alter the isoelectric point and/or molecular mass of the proteins. Some post-translational modifications affect the charge on the protein and the ability of a given protein to pass through the glomerular permeability barrier. As many plasma proteins exist in a variety of *N*-glycosylated states, some of which have different charges, they may be filtered differentially. 2DE cannot be applied to peptides <10 kDa, or it cannot be automated. Further, it is time-consuming, and is difficult for quantitative analysis.

Recently, the concept of two-dimensional difference in-gel electrophoresis (2D-DIGE) was introduced to reduce gel-to-gel variability [7]. Two samples are differentially labeled with fluorescence dyes, and the two samples are then resolved simultaneously within the same 2DE gel. This technique also allows introduction of an internal standard labeled with a third dye, allowing quantitative analysis.

### 2.2. Mass spectrometry (MS)

The identification of proteins is achieved by MS. Matrix-assisted laser desorption/ionization (MALDI) and electrospray ionization (ESI) are the most commonly used methods to volatilize and ionize proteins for MS analysis. There are different basic types of mass analyzers used in protein analysis, including time-of-flight (TOF), quadrupole (Q), ion trap (IT), Fourier transform ion cyclotron resonance (FTICR), and magnetic type. MALDI is usually coupled with a TOF analyzer, whereas ESI is usually coupled with triple quadrupole (QQQ) or IT. Tandem mass spectrometry (MS/MS) is a technique that permits identification of peptide sequence by generating product ion spectra of a selected precursor ion, includes QQQ, TOF/TOF, Q/TOF, and Q/IT analyzers. HPLC or CE can be directly combined with MS or MS/MS, the ionization of which is ESI. The combination of HPLC or CE with MS/MS enables identification of specific proteins in a complex mixture without the need for initial preseparation of the individual proteins. The use of MS for the study of clinical medicine, uremic toxins and post-translational modification of proteins has been reviewed [8–11].

The first step in the process of identifying proteins in the 2DE gel spots by MS is a proteolytic in-gel digestion by exposing the excised pieces of gel to trypsin followed by extraction of the proteolytic fragments from the gel. The fragments are subsequently analyzed by MS. The identification of a specific protein by MS and trypsin digestion can be achieved by two approaches. The first one is peptide mass fingerprinting by use of MALDI-TOF-MS, and the second one is peptide sequencing by MS/MS. Mass fingerprinting involves the molecular masses of all the trypsin products. Trypsin cleaves after lysine and arginine. The measured masses of the peptides are matched by computer analysis with the theoretical masses of the trypsin products of all the known protein sequences. Peptide sequencing using MS/MS identifies a specific protein by measuring product ion spectra of the selected precursor ion of the peptide. The difference in $m/z$ between successive product ions that differ by the mass of an amino acid identifies the peptide sequence.

### 2.3. Surface-enhanced laser desorption/ionization time-of-flight mass spectrometry (SELDI-TOF-MS)

SELDI-TOF-MS technology uses protein-chip arrays coupled with a TOF mass spectrometer. The chip solid-phase surface is activated chemically or biochemically. Proteins bind to a specific surface (hydrophilic matrix, reverse-phase material, or antibodies) with varying degrees of selectivity while the unbound sample is washed away. A matrix that allows vaporization and ionization of the sample by laser is added on the sample surface. The sample is subsequently analyzed by laser desorption/ionization MS that identifies individual proteins as separate peaks on the basis of their *m*/*z*. Because SELDI-TOF-MS is easy to use, it can be automated, and can analyze multiple samples in a short time (high throughput). Therefore, SELDI-TOF-MS has been used to discover biomarkers for a variety of diseases. However, it is restricted to selected proteins, and the mass spectra obtained are of low resolution. A small fraction of all proteins in a sample binds to the chip surface, and the binding varies depending on sample concentration, pH, salt content, and on the presence of interfering compounds such as lipids.

### 2.4. Liquid chromatography/electrospray ionization mass spectrometry (LC/ESI-MS)

Liquid chromatography (LC) provides a powerful fractionation method that is compatible with virtually any mass spectrometer with ESI. This method separates large amounts of analytes on a HPLC column with high sensitivity, and can be automated. A sequential separation using different media in two independent steps provides a multi-dimensional fractionation that can generate huge amounts of information. The multi-dimensional protein identification technology and a two-dimensional liquid-phase fractionation approach are well suited for exact analysis of body fluids and tissues. However, LC/ESI-MS is time-consuming, and sensitive to interfering compounds. Because high-molecular-weight proteins cannot be analyzed by LC/ESI-MS, they should be cleaved by trypsin, and the resulting fragments analyzed.

### 2.5. Capillary electrophoresis/electrospray ionization mass spectrometry (CE/ESI-MS)

This approach is based on capillary electrophoresis (CE) at the front end coupled to a mass spectrometer with ESI. CE separates proteins in a single step with high resolution based on their migration through a gel in an electrical field. CE/ESI-MS provides fast and high-resolution analysis using an inexpensive capillary, is compatible with most buffers and analytes, and can be automated. CE/ESI-MS enables the generation of high-resolution data. The data from individual analyses can be compiled to generate a typical proteome pattern that can be based on >100 individual analyses. However, CE/ESI-MS is not suited for the analysis of high-molecular-weight proteins >20 kDa. Such proteins can be digested and the resulting fragments can be analyzed. Furthermore, they can be effectively removed by ultrafiltration. In addition, certain proteomes, such as the urinary proteome of healthy individuals, contain mostly low-molecular-weight proteins; in such cases, the restricted ability to analyze large native proteins does not constitute severe drawback. Sequencing of potential protein biomarkers that are defined by CE/ESI-MS can be achieved by CE/ESI-MS/MS instruments or by subsequent targeted sequencing using LC/ESI-MS/MS. CE/ESI-MS has been clinically applied for the diagnosis of kidney diseases, urogenital cancer, and arteriosclerosis as well as monitoring the responses to therapeutic interventions [2].

### 2.6. Imaging mass spectrometry (IMS)

IMS is a technique for the direct analysis of peptides and proteins from thin tissue sections using MALDI-TOF-MS, while preserving the abundance and spatial distribution of each analyte. IMS represents a new analytical tool to directly provide the spatial distribution and the relative abundance of proteins in tissue [12].

## 3. Protein biomarkers for kidney disease

### 3.1. Primary glomerular disease

Park et al. [13] used 2DE and MALDI-TOF-MS to establish the urinary protein map of IgA nephropathy. A large number of protein spots were identified in patients with IgA nephropathy and normal control samples, with means of 311 spots and 174 spots, respectively. Approximately 216 protein spots were detected as differentially expressed in IgA nephropathy. A total of 84 differentially expressed spots, representing 59 different proteins, were finally identified in IgA nephropathy.

Varghese et al. [14] also used 2DE and MALDI-TOF-MS to identify characteristic patterns of urinary proteins for kidney diseases. Urine proteins obtained from patients with focal segmental glomerular sclerosis, lupus nephritis, membranous nephropathy, and diabetic nephropathy, were separated by 2DE. Twenty-one gel spots were most important for the differentiation of the diseases. Twenty spots were identified using MALDI-TOF-MS as charge forms of the following eleven plasma proteins: orosomucoid, transferrin, α-1-microglobulin, zinc α-2-glycoprotein, α-1-antitrypsin, complement factor B, haptoglobin, transthyretin, plasma retinol binding protein, albumin, and hemopexin. Diseases that induce nephrotic syndrome change glomerular protein permeability in characteristic patterns. The fingerprint of urine protein charge forms can identify the glomerular disease. These proteins are candidate biomarkers for the diagnosis of kidney diseases.

Khurana et al. [15] used SELDI-TOF-MS to identify urinary biomarkers of steroid-resistant nephrotic syndrome of childhood. Urine samples were obtained from pediatric and adolescent patients with steroid-sensitive nephrotic syndrome, and steroid-resistant nephrotic syndrome. Five peaks with *m*/*z* values of 3917.07, 4155.53, 6329.68, 7036.96, and 11,117.4, could distinguish steroid-resistant nephrotic syndrome. Especially, the peak at *m*/*z* 11,117.4 was demonstrated to be a biomarker of

steroid-resistant nephrotic syndrome with an accuracy of 95%, and was consequently identified as β2-microglobulin.

Woroniecki et al. [16] also used SELDI-TOF-MS to discover urinary biomarkers for steroid-resistant nephrotic syndrome of childhood. A protein of mass 4144 Da was identified as the single most important classifier in distinguishing steroid-sensitive nephrotic syndrome from steroid-resistant nephrotic syndrome.

Chalmers et al. [17] used CE/ESI-TOF-MS for urinary biomarker discovery and structural characterization, based on both top–down and bottom–up analyses to separate and measure the thousands of polypeptides. Statistical analysis of the differences between healthy subjects and patients with focal segmental glomerulosclerosis, membranous nephropathy, minimal change disease, IgA nephropathy, and diabetic nephropathy validated multiple biomarkers for the diseases.

### 3.2. Diabetic nephropathy

Jain et al. [18] applied 2DE and MALDI-TOF-MS to identify urinary protein markers for specific and accurate prediction of nephropathy in type 2 diabetic patients. Four main proteins along with albumin were identified as zinc α-2 glycoprotein, α-1 acid glycoprotein, α-1 microglobulin and IgG in the urine of diabetic patients with microalbuminuria. These proteins can be used as markers for specific and accurate clinical analysis of diabetic nephropathy.

Kim et al. [19] used 2DE and ESI-Q-TOF-MS/MS to discover serum protein biomarkers for accurate prediction of progressive diabetic nephropathy in serum from type 2 diabetic patients. Extracellular glutathione peroxidase and apolipoprotein E exhibited a progressive reduction in microalbuminuria and chronic renal failure groups, and may be potential biomarkers for the diagnosis of type 2 diabetics with nephropathy.

Rao et al. [20] used 2D-DIGE and LC/ESI-MS/MS to identify urinary protein biomarkers of diabetic nephropathy in urine from type 2 diabetic patients. One hundred and ninety-five protein spots were detected representing 62 unique proteins. Seven proteins (α-1B-glycoprotein, zinc-α-2-glycoprotein 1, vitamin D-binding protein, α-2-HS-glycoprotein, calgranulin B, α-1-antitrypsin, and hemopexin) were progressively up-regulated with increasing albuminuria, and four proteins (retinol binding protein, α-1-microglobulin/bikunin precursor, apoA-1, and transthyretin) were progressively down-regulated. Thus, these proteomic analyses were useful to detect potential urinary biomarkers for diabetic nephropathy.

Mischak et al. [21] used CE/ESI-MS to identify urinary biomarker for early detection of diabetic renal damage. CE/ESI-MS enabled fast and accurate identification and differentiation of polypeptide patterns in urine of type 2 diabetic patients. In diabetic patients with normoalbuminuria, the urinary polypeptide pattern differed significantly from normal, indicating a specific diabetic pattern of polypeptide excretion. In patients with higher grade albuminuria, a polypeptide pattern indicative of diabetic renal damage was detected. Several of the indicative polypeptides were identified using MS/MS peptide sequencing.

Rossing et al. [22] also used CE/ESI-MS to evaluate urinary polypeptide patterns and the effect of angiotensin II receptor blocker candesartan on the patterns in type 2 diabetic patients with normoalbuminuria, microalbuminuria, and macroalbuminuria. Differences in urinary polypeptide patterns between normo- and macroalbuminuric patients permitted the establishment of a diabetic renal damage pattern consisting of 113 polypeptides. Candesartan treatment in macroalbuminuric patients changed 15 of the 113 polypeptides in the diabetic renal damage pattern toward levels in normoalbuminuric patients.

### 3.3. Lupus nephritis

Mosley et al. [23] applied SELDI-TOF-MS to identify urinary biomarkers for diagnosis of lupus nephritis and monitoring of its activity and response to therapy. Proteins with masses of 3340 and 3980 could distinguish active from inactive lupus nephritis. Identification of these proteins will lead to the development of specific assays to monitor disease progression.

### 3.4. Acute renal allograft rejection

The diagnosis of renal allograft rejection requires a renal biopsy at present. Clinical management of renal transplant patients would be improved by the development of non-invasive biomarkers of rejection. Schaub et al. [24,25] used SELDI-TOF-MS to discover such candidate proteins in urine, and demonstrated that they derived from non-tryptic cleaved forms of β2-microglobulin. In-vitro experiments showed that cleavage of intact β2-microglobulin requires a urine pH less than 6 and the presence of aspartic proteases. Patients with acute tubulointerstitial rejection had lower urine pH than stable transplants and healthy subjects. They showed high amounts of aspartic proteases and intact β2-microglobulin in urine. These factors ultimately lead to increased levels of cleaved urinary β2-microglobulin. Cleaved β2-microglobulin may become a useful tool as an indicator of acute tubular injury for non-invasive monitoring of renal allografts.

Oetting et al. [26] used MALDI-TOF-MS to identify urinary biomarkers of acute rejection of kidney allografts. A urinary protein peak at 11.7 kDa that was identified as β2-microglobulin, correlated strongly with acute rejection. Thus, β2-microglobulin is a biomarker for acute rejection.

### 3.5. Acute kidney injury

Nguyen et al. [27] used SELDI-TOF-MS to identify urinary biomarkers for ischemic kidney injury. Protein biomarkers with *m/z* of 6.4, 28.5, 43 and 66 kDa were markedly enhanced in urine from acute renal failure patients at 2 and 6 h post-cardiopulmonary bypass compared with baseline. The sensitivity and specificity of the 28.5-, 43- and 66-kDa biomarkers for the prediction of acute renal failure at 2 h following cardiopulmonary bypass was 100%.

Urinary exosomes are obtained by ultracentrifugation of urine, and are small membrane vesicles with diameter less than 80 nm that originate as internal vesicles of multivesicular bodies. The exosomes are derived from epithelial cells, are normally secreted into urine from all nephron segments, and

contain both membrane proteins and cytosolic proteins. Pisitkun et al. [28] used nanospray LC/ESI-MS/MS to analyze the exosomes in normal human urine, and identified numerous protein components of multivesicular bodies and endosomal pathway. Two hundred and ninety-five proteins were detected, including multiple protein products of genes already known to be responsible for renal and systemic diseases, including autosomal dominant polycystic kidney disease, Gitelman syndrome, Bartter syndrome, autosomal recessive syndrome of osteopetrosis with renal tubular acidosis, and familial renal hypomagnesemia. Thus, exosomes contained many disease-associated proteins including aquaporin-2, polycystin-1, podocin, nonmuscle myosin II, angiotensin-converting enzyme, $Na^+$ $K^+$ $2Cl^-$ cotransporter (NKCC2), thiazide-sensitive Na-Cl cotransporter (NCC), and epithelial sodium channel (ENaC). Urinary exosomes are advantageous as a starting material for biomarker discovery, and a strategy for biomarker discovery in urine using exosomes has been reviewed [29].

Zhou et al. [30] used 2-DE, MALDI-TOF/TOF-MS and LC/ESI-MS/MS to identify urinary protein biomarkers of acute kidney injury in exosomes. Cisplatin was injected into rats to induce kidney injury. Urinary exosomes were isolated by differential centrifugation. Exosomal fetuin-A was increased 52.5- to 51.5-fold at days 2–5 after cisplatin injection. Urinary exosomal fetuin-A was increased in three patients on intensive care with acute kidney injury compared to the patients without acute kidney injury. Thus, urinary fetuin-A may be a biomarker of acute kidney injury.

Potential toxicity biomarkers in kidneys of gentamicin-administered rats were searched using IMS [12]. Differential analysis of the mass spectrum profiles revealed a spectral feature at 12,959 Da that strongly correlates with histopathology alterations of the kidney. The protein of 12,959 Da was identified as transthyretin (Ser(28)-Gln(146)) using an combination of tissue microextraction and fractionation by reverse-phase HPLC followed by a top–down MS/MS. IMS plays an exciting role in the discovery of toxicity biomarkers in tissues.

### 3.6. Fanconi syndrome

In renal Fanconi syndromes, such as Dent's disease, urinary peptides are implicated in altered tubular function or injury. Cutillas et al. [31] used nanoflow LC/ESI-MS/MS to analyze urinary peptides in patients with Dent's disease. Urine was desalted by solid-phase extraction and its peptides were then separated from neutral and acidic compounds by strong cation-exchange chromatography. Fractions from the strong cation-exchange step were separated further by LC/ESI-MS/MS. Over 100 molecular species were detected in the urine samples.

### 3.7. Renal cell carcinoma

Urinary proteins of molecular masses <30 kDa were fractionated from larger proteins. The fraction containing proteins with molecular masses >30 kDa was subsequently subjected to immunoaffinity subtraction chromatography removing most of abundant albumin and IgG. 2DE and subsequent MS analysis of about 1400 spots using MALDI-TOF-MS peptide mass fingerprinting and LC/ESI-MS led to the successful identification of 30% of the proteins [32]. Because of post-translational modifications in most urinary proteins and the presence of proteolytic products, 420 identified spots collapsed into 150 unique protein annotations. The differential display of urinary proteins from a renal cell carcinoma patient before and after nephrectomy revealed a marked decrease in mannan-binding lectin serine protease 2, and more dramatically, kininogen after nephrectomy.

## 4. Conclusion

The diagnosis of kidney diseases requires renal biopsy that is an invasive technique with some inevitable risk. Therefore, non-invasive diagnosis of kidney diseases is a challenge in clinical nephrology. Recent advances in proteomics may enable the non-invasive diagnosis of kidney diseases by identifying protein biomarkers in urine. Many biomarker candidates have been identified using the proteome MS analysis of urine. These biomarker candidates should be validated in a number of patients if they are clinically useful to diagnose the kidney diseases, to predict the prognosis and to monitor the effects of treatment.

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