

LECTURE-35

URINE PROTEOMICS

HANDOUT

PREAMBLE

The comparison of protein expression patterns in biological fluids of healthy individuals and patients is used for the discovery of biomarkers as well as determination of biochemical processes influencing disease pathogenesis. Various methodologies that are employed in urine proteomic studies, majorly work on measuring the total level of proteins or the differential expression of proteins between samples that are obtained from different sources. The advancement in proteomic techniques has allowed the concomitant analysis of patterns of multiple urinary proteins and their connection with individual diagnoses and facilitated closer monitoring of responses to treatment or prognosis.

OUTLINE OF LECTURE

- I. Introduction
- ii. Sample collection
- iii. Techniques used in urine proteomics
- iv. Clinical applications
- v. Challenges
- vi. Conclusions

BOX-1: TERMINOLOGY

- **Proteomics:** The study of entire complement of proteins expressed by the genome of an organism under specific defined conditions is known as proteomics.
- **2-DE:** Two Dimensional Gel Electrophoresis is a protein profiling technique that carries out separation using isoelectric focusing in first dimension, followed by SDS-PAGE in the second dimension.
- **2D-DIGE:** 2D Difference in Gel Electrophoresis is an advanced form of 2-DE that allows simultaneous analysis of test and control samples on a single gel by carrying out differential labeling of each sample. This minimizes gel-to-gel variations and enables easy processing of large number of samples.
- **SELDI-MS:** Surface-Enhanced Laser Desorption/Ionization coupled to mass spectrometry is a technique, which facilitates selective binding of proteins under the influence of special parameters of pH, temperature, etc. to the surface. A matrix material is then mixed on to the surface, which is further ionized and detected by mass spectrometry.
- **CE-MS:** Capillary Electrophoresis coupled to Mass Spectrometry is the technique wherein protein separation occurs with high-resolution based on their migration through a buffer-filled capillary column in an electrical field and is further detected by mass spectrometry.

I. INTRODUCTION

Physicians have used urine analysis as an effective tool for disease assessment since decades. The qualitative and quantitative analysis of proteins present in urine can be used for gaining insights into the disease pathogenesis. Proteomic techniques also hold great promises in development of novel and non-invasive diagnostic tools, majorly with respect to nephrological disorders. In normal situations, the excretory system of humans functions in such a way that the low-molecular-weight proteins and albumin are filtered from plasma and are almost completely re-absorbed. Thus the proteins that are found to compose the urine are mainly the ones which are results from the events of blood plasma's glomerular filtration, cell sloughing, programmed cell death, enzyme-mediated cleavage of cell surface proteins or because of secretion of exosomes by the epithelial cells. The total protein count in the urine reaches to an average of 1000. Consequently, the daily urinary protein excretion in healthy individuals is less than 150 mg/day, of which about 10 mg is albumin. In the case of patients suffering with physiological proteinuria; mucoproteins, blood-group proteins, albumin, immunoglobulins, mucopolysaccharides, and very small amounts of hormones and enzymes, are found to be excreted in the urine. Initially, proteinuria of more than 150 mg/day was regarded as abnormal. The low levels of albumin (~30 mg/day), which is exhibited in early stages of renal disease is termed as "microalbuminuria". Protein or albumin excretion greater than 300 mg/day is termed as "macroalbuminuria". Urine proteomics can be used as a good indication of renal disorders and can have pathophysiological implications as well. On the other hand, urine sample is highly variable with respect to its composition, which

further gets magnified at the proteomic level. These differences can however be compensated by standardization based on urinary creatinine or urinary housekeeping peptides, which are present almost ubiquitously in human urine. Furthermore, healthy kidney tubular physiology is reflected by presence of normal urinary proteins as urine contains not only plasma proteins but kidney proteins as well. Urine can thus be regarded as a good source for the study of processes that affect proximal organs, including kidney failure, and diabetic nephropathy.

II. SAMPLE COLLECTION

Urine proteomics has gained wide-scale application since sample procurement is easy and non-invasive. Besides that, urine can be obtained in large amounts and it is also a source of numerous proteins and peptides, which are less complex and can be detected easily through biochemical assays. The concentration of proteins in urine is very low and hence it requires concentration steps prior to analysis. Approximately 0.5 mg of proteins can be obtained from a single sample. Collection of midstream urine as compared to the first-void urine is always considered as the standard for almost all urine analysis. Urine samples are collected in urine collection bags and for a limited time, it can be stored at 4°C, while for a relatively long time, it is best to freeze at –80°C before analysis. This is advantageous as urine can be collected any time and over lengthy time periods.

AN OVERVIEW OF STEPS INVOLVED IN URINE PROTEOMIC ANALYSIS

- Sample collection and storage
- Protein extraction
- Gel-based - First dimension Isoelectric Focusing; Second dimension SDS-PAGE; Staining and destaining; Data analysis and determining differential expression of proteins; spots excision, in-gel tryptic digestion
- Gel-free – iTRAQ labeling; in-solution tryptic digestion
- Identification, validation and clinical application of identified significant proteins as putative biomarkers

III. TECHNIQUES USED IN URINE PROTEOMICS

A combination of different protein concentration and separation techniques has been widely used for urine proteomic analysis. Some of the commonly used techniques are described in this section.

(A) Two-dimensional gel electrophoresis coupled with MS

2-DE was first reported for protein separation by O'Farrell (1975) and bears wide applications till today. In this technique, the proteins are separated in the first dimension by Isoelectric Focusing (IEF) and then in second dimension by Sodium Dodecyl Sulphate- Polyacrylamide gel electrophoresis (SDS-PAGE). Post-separation, the proteins are stained and then analyzed from software for understanding different protein expression patterns. Individual proteins are in-gel digested and further identified by Mass Spectrometry. 2-DE is however a time-consuming and laborious procedure and has the major disadvantage of low reproducibility due to gel-to-gel variation. Moreover, with 2-DE, it is difficult to analyze smaller proteins (<10 kDa) and highly hydrophobic proteins. As an improvement to alleviate the limitations associated with 2-DE, the concept of Two-dimensional difference gel electrophoresis (2D-DIGE) was introduced, wherein two urine proteome samples are differentially labeled with fluorescence dyes (cy3 and cy5) and mixed together and then separated within the same 2-DE gel. An internal standard labeled with a third dye (cy2) is also incorporated, thereby allowing more accurate quantitative analysis for urine proteome.

(B) Surface-Enhanced Laser Desorption/Ionization Coupled to mass spectrometry (SELDI-MS)

For proteomic analysis of complex samples like Urine, SELDI can prove to be advantageous because it reduces the complexity of biological samples by selective interactions of polypeptides with different active surfaces, which are hydrophilic and allow reverse-phase interactions. After the interaction phase, only a small fraction of polypeptides can bind to the surface of the SELDI chip, depending on factors like the concentration, pH, salt content, presence of interfering compounds like lipids, etc. The unbound sample is eliminated in the consequent washing steps. A matrix material is added to the sample surface to absorb energy to allow vaporization and ionization by laser for further MS detection. A variety of disease biomarkers have been identified by this technique because of its ease of operation and high throughput efficiency. However, this technique has certain limitations with respect to the reproducibility as variations are introduced due to different chip surfaces and conditions. The multi-dimensional fractionation technique when adopted using sequential separation of different matrices in two independent steps is capable of generating large amount of data, which when compiled together can provide large amount of complementary information.

(C) Capillary Electrophoresis Coupled to Mass Spectrometry (CE-MS)

CE-MS is a widely used MS-based approach for the proteomic analysis of body fluids such as urine. This approach is based on CE as a front end fractionation device, which is further coupled to a mass spectrometer. In CE, protein separation occurs with high-resolution based on their migration through a buffer-filled capillary column in an

electrical field (300 to 500 v/cm). CE stands out to be an advantageous technique because of its fast and robust separation and resources required are inexpensive as well. Furthermore, since the technique is compatible with most buffers and analytes, it can be used effectively for urine proteomic studies.

(D) Protein Microarrays

Protein microarrays can be used for proteome analysis on complex samples like urine. In this technique, specific antigens or antibodies are printed onto glass substrate or a membrane. The immobilized entity is then allowed to undergo complementary hybridization with the proteins present in the sample. The captured antigens or antibodies are then subsequently detected colorimetrically or through fluorescence. Microarrays are highly advantageous with respect to their sensitivity and allows for simultaneous detection of multiple proteins. A small limitation with this technique is the requirement of highly specific proteins or antibodies for detection of certain proteins.

IV. CLINICAL APPLICATIONS

URINARY PROTEOMICS FOR BIOMARKER DEVELOPMENT

The process of biomarker development mainly comprises of the following three stages:

- A) Discovery of presumptive biomarkers.
- B) Validation of these markers in terms of their ability to make significant predictions with respect to disease diagnosis, their sensitivity and specificity.
- C) Implementation with respect to development of a related clinical assay.

BIOMARKER DEVELOPMENT FOR DIABETIC NEPHROPATHY (DN)

Diabetic nephropathy is a serious complication of diabetes and is observed in diabetics at a later stage of progression. Traditionally, renal biopsy and albumin levels have been used for diagnosis of diabetic nephropathy. However, these methods are invasive and take a back seat when it comes to sensitivity and specificity. This makes it necessary to develop biomarkers, which are non-invasive and have high sensitivity and specificity for effective disease diagnosis and treatment. A study by Jiang et al. 2009 analyzed type 2 diabetic patients with normoalbuminuria, type 2 diabetic Nephropathy patients, and health subjects. By involving 2-DE and mass spectrometry authors identified soluble E-cadherin as a novel biomarker for diabetic nephropathy.

URINARY PROTEOMICS IN CANCER DIAGNOSIS

The detection of cancer at the very primitive stage is seen as one of the prime goals of clinical medicine. Urine proteomics because of its wide scope can provide promising tools, which can be of use in diagnosis of not only cancer but also other urological and systemic malignancies. Bladder cancer stands out to be the most widely understood

disease through urine proteomic studies. Majority of the bladder cancers have been analyzed by 2-DE analysis of tumor tissue belonging to the epithelium that lines the urinary drainage system. The protein expression patterns are compared with that of healthy individuals and of patients suffering from other types of bladder cancer, for instance the squamous cell carcinoma. Experimental results have identified psoriasin as a potential biomarker which is found to be present in urine of patients who are suffering from squamous cell carcinoma. On comparative analysis, it was found to be less frequent in urine samples of patients suffering from transitional cell carcinoma. Apart from this, cytological studies of the urine have also been used in combination with proteomic studies to establish confirmatory results. Besides this, the degree of progression of the tumor is studied on the basis of amounts of adipocyte-fatty acid-binding protein expression levels; which are found to be in lower levels in case of patients suffering with high-grade transitional cell carcinoma. This has been verified through 2-DE and immunoblotting techniques. The field of cancer proteomics holds bigger promises by analyzing urinary proteome.

URINE PROTEOMICS IN PREGNANCY DETECTION TESTS

The presence of hormone Human chorionic gonadotropin in urine of females has been traditionally understood as a positive indication of pregnancy. This pregnancy test makes the use of the principle binding mechanism between the hormone and two monoclonal antibodies, which are impregnated on the test strip. One of the two monoclonal antibodies is conjugated with gold. The interaction between the two entities is detected as color reaction develops. This test is highly sensitive, cheap and of technical ease to the user.

CLINICAL APPLICATION	TECHNIQUES USED	KEY FINDING	REFERENCES
Diagnostic marker for detection of Immunoglobulin A Nephropathy (IgAN)	Capillary Electrophoresis-MS	IgAN-specific urinary polypeptide pattern which helps in distinction of IgAN and membranous nephropathy	Haubitz et al. 2005
Acute kidney injury/renal failure (AKI)	SELDI in combination with MS-MS	Aprotinin identified as a marker for AKI	Nguyen et al. 2005
Structural Renal Injury	MALDI-TOF-TOF-MS and further validation by Western Blot Analysis	Urinary fetuin-A identified as a biomarker for structural renal injury	H Zhou et al. 2006
Acute Renal Allograft Rejection	SELDI and Capillary Electrophoresis in combination with MS-MS	Proteolytic products of Microglobulin	Rose et al. 2006

BOX-2: URINARY PROTEOMICS - KEY POINTS

- Protein excretion in urine of patients suffering from intrinsic renal disease or acute nephrological pathophysiologies can provide information, which holds importance for the diagnostic and prognostic perspective.
- Urine proteomic studies have aided in identification of numerous novel urine proteins that can facilitate diagnosis as well as monitoring of renal and systemic diseases.
- Integration of urine biomarkers in routine clinical practice is highly required; however, it is likely that multiple biomarkers will be needed to provide the sensitivity and specificity necessary for their use in clinical decision-making.

V. CHALLENGES

Urine is a highly complex body fluid comprising of numerous proteins in normal healthy individuals. This makes it difficult to delineate specific biomarkers for diseased conditions. Therefore, it becomes necessary that the proteomic tools that are employed should be highly sensitive and specific. Besides that, since urine proteomics holds wide applications in the field of clinical diagnostics, it is essential that the techniques used should be highly reproducible. This shall ensure proper diagnosis and efficient disease management and therapeutic monitoring. Moreover, the field of urine proteomics currently bears the challenge that different forms of renal and extrarenal disease be distinguished from each other. This is important because in certain cases, excretion of some urinary proteins may be considered as a false positive sign for a diseased condition. There are few factors that play an important role for urine proteome analysis such as time at which the urine sample is collected, the conditions in which it is stored and the need for normalization between different sources of urines for effective data analysis. The combined efforts of multiple groups intersecting at the platform created by Human Kidney and Urine Proteome Project (www.hkupp.org/), under the umbrella of the World Human Proteome Organization (www.hupo.org/) is presently addressing these challenging issues and is instrumental in publishing standardized methods for urinary proteomic studies.

VI. CONCLUSIONS

The wide-scale applications of urine proteomics hold great significance in the fields of urology, nephrology and oncology. The discovery of various biomarkers, which are characteristic of not just a particular pathophysiological condition but also indicative of the degree of a particular disease spread has opened up a new scope for clinical diagnostics. The advancement of various proteomic technologies has accelerated the pace of urine proteomic research. The urine proteomics can be explored for the discovery and validation of urinary biomarkers and these efforts shall further aid to understand the disease prognosis and therapeutic interventions.

REFERENCES

1. Haubitz, M., Wittke, S., Weissinger, E.M., Walden, M., Rupprecht, H.D., Floege, J., Haller, H., Mischak, H., Urine protein patterns can serve as diagnostic tools in patients with IgA nephropathy. *Kidney Int.* 2005 Jun;67(6):2313-20.
2. Nguyen, M.T., Dent, C.L., Ross, G.F., Harris, N., Manning, P.B., Mitsnefes, M.M., Devarajan, P., 2008. Urinary aprotinin as a predictor of acute kidney injury after cardiac surgery in children receiving aprotinin therapy. *Pediatr Nephrol.* 2008 Aug;23(8):1317-26.
3. Zhou H, Pisitkun T, Aponte A, Yuen PS, Hoffert JD, Yasuda H, Hu X, Chawla L, Shen RF, Knepper MA, Star RA. Exosomal Fetuin-A identified by proteomics: A novel urinary biomarker for detecting acute kidney injury. *Kidney Int.* 2006 Nov;70(10):1847-57.
4. Pisitkun T, Johnstone R and Knepper RA, Discovery of Urinary Biomarkers. 2006. *Molecular and Cellular Proteomics.* 5, 1760-1771.
5. Barratt J and Topham P, Urine proteomics: the present and future of measuring urinary protein components in disease. 2007. *CMAJ.* 2007 August 14; 177(4): 361–368.
6. Jiang H, Guan G, Zhang R, Liu G, Cheng J, Hou X, Cui Y. Identification of urinary soluble E-cadherin as a novel biomarker for diabetic nephropathy. *Diabetes Metab Res Rev.* 2009 Mar;25(3):232-41. doi: 10.1002/dmrr.940.