Youhe Gao *Editor*

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Promising Biomarker Source for Early Disease Detection

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Theory, Strategy and Tools

Chapter 1
Urine Is Not a Human Waste but a Medical Treasure

Youhe Gao

Abstract A biomarker is a measurable change associated with the biological process (including continuously from healthy normal stage to disease stage). Controlled by homeostatic mechanisms of the body, all cell bathing in body fluids, mainly blood and cerebral spinal fluid, tend to remove early changes. Wastes of the body, such as urine and breath, accumulating all kinds of early changes, are better biomarker sources.

Keywords Urine · Early disease biomarker · Next-generation biomarker

We need water and food to survive. The second thing we care about is health. To keep healthy, we need to know the health status of the body and stop or at least slow down the disease process before it becomes irreversible. So far as the most studied biomarker source, blood fails to meet that need. We need earlier biomarkers, much earlier than the current ones. I want to name it the next-generation biomarker. Early has been used so much in literature; we do not know what is actually early. There are multiple ways to define early. Before curable clinically is usually considered early enough practically. But from the disease development point of view, there are other time points. Two are probably easy to monitor. One is before pathological changes under light microscope. This is easy to use in animal model studies. Another is before there is any blood test abnormality. This is better for clinical use since it is almost harmless.

Here we want to emphasize that there are other time points even before these two during the disease development when we can noninvasively check the body's health status, if we monitor urine. In other words, I want to say urine is the source of next-generation biomarker source.

We cannot discuss urine as a better biomarker source without touching the definition of biomarker. There were many biomarker definitions already. I think to find the most accurate one, we need to think about step by step how we have been doing biomarker studies. We always used at least two groups of samples to compare, healthy vs disease, type I vs type II, in situ vs metastasis, and drug sensitive vs drug resistant.

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No matter what parameters we measure in these samples, we always compare by subtraction or division. By nature, we looked for the distance, the difference or say changes. I personally like the word change since it emphasizes the disease development. With this analysis we may agree now that a biomarker is a measurable change associated with a biological process or say a disease. The key word in this definition is CHANGE. Searching for a biomarker is looking for changes (as disease develops). Where should we be able to find subtle changes at the very beginning of the disease development (somebody may not agree to call it a disease yet clinically)?

Let's review the beautiful theory of homeostasis. Please allow me to quote from Wikipedia "The concept of the regulation of the internal environment was described by French physiologist Claude Bernard in 1865, and the word homeostasis was coined by Walter Bradford Cannon in 1926 (Cannon 1926, 1939). Homeostasis is an almost exclusively biological term, referring to the concepts described by Bernard and Cannon, concerning the constancy of the internal environment in which the cells of the body live and survive" (Cannon 1926, 1939; Zorea 2014). Blood is part of the internal environment. So, blood tends to be stable. Blood is kept stable by many organs of the body including the kidney, lung, liver, and skin. Small changes associated with the early disease development are removed by those organs from the blood. If small changes are removed from the blood at the early stage of the disease, how could we find the early disease biomarker from blood? Even with so many brilliant scientists and engineers worked so many years so diligently with so much money, so far there is still not enough satisfactory finding. We always think if the machines we used are a little more sensitive, we may get some good biomarkers in blood. Actually, if we changed the places to search, we may already have some good early biomarkers even with current technology.

To summarize the above analysis, the biomarker theory goes like this: (1) A biomarker is a measurable change associated with the biological process (including continuously from healthy normal stage to disease stage); (2) controlled by homeostatic mechanisms of the body, all cell bathing body fluids mainly blood and cerebral spinal fluid tend to remove early changes; (3) wastes of the body such as urine and breath accumulate all kinds of early changes and are better biomarker sources (Gao 2013).

People may wonder without this theory; many scientists had been working on urine and breath for many years. The results were not considered even as good as those from blood. What happened?

Let's think a little further down the road. This biomarker theory says that early subtle changes are in urine. It shows us the promising side of the fact that we can find early biomarker in urine. But it concealed the troubling side of the fact that there are so many changes in urine which may not even relate to the disease we study. They are from other confounding factors, such as different climates, food, drinks, lifestyles, supplements, and medications. If we neglected those factors, we might not find the exact early disease biomarker. What we found were the effects of all the factors combined (Gao 2014a). When other factors were not controlled, the findings would be hard to verify. It is possible to discover early disease biomarkers if we carefully control all the factors. But with that many factors to control at the

same time, we need to have a huge number of samples to stratify. The cost is tremendous. Many of the urine biomarker researchers might not be able to commit such amount of resources, especially when we were not sure if there is a good early biomarker in urine.

I think the most important and urgent thing in biomarker field is to convince people that there are good early biomarkers in urine. To convince other people without being broke first, we need to reduce the number of confounding factors to minimum.

The roadmap is using animal models to minimize the confounding factors and to reproduce the very early disease situation, discover the changes in urine (or other wastes), and then verify in clinical samples later (Gao 2014b).

The disease process of the animal model can be monitored at the very beginning of the disease. It is much earlier than the chief complains from the patient or than the clinically defined early. Since there are very few confounding factors, even though the disease may not exactly be the same as human disease, I still think the results from animal model studies may be easier to be recognized as the evidence that the disease can be reflected very early in urine. I understand that I am overoptimistic since the results are just too good to be considered as true.

The animal model results keep coming in the laboratory every month. Some of them are included in this book. I hate to reiterate all the results and steal the show which belongs to all my great lab members. I do not want to gamble which candidate biomarker is more promising than the other. But I do want to make one bet from all those results. It seems to me that almost if not all the diseases from almost if not all organs of the body can be reflected in urine, and at a very early stage.

I actually have a shortcut to skip the above proposed roadmap. If I have enough clinical resources, I might have already tried it. The shortcut is to skip the collection of candidate biomarkers information from animal model(s); instead, extract that information from existing studies. Those candidate biomarkers can be differential proteins in tissues, in any body fluids, or even proteins known to relate to disease mechanisms. We simply collect all the information of them and test if some of them can be present in urine in the early stage of the disease. Bingo if some of them are (Gao 2014c).

There is a major difference between urine biomarker and blood/CSF biomarker. Blood and CSF have relatively stable volumes. Concentration change is consistent with its quantity change in the body. But urine volume can be easily changed as the volume of water drinking varies. For the same quantity changes of a specific material in the body, its concentration in urine will go down as the water intake increases. This is why urine biomarker should never use concentration as the unit. The more stable measurement of a particular material change is probably its ranking compared to other materials in urine. In normal water drinking range, rankings of most proteins are likely to be stable. As more water intake, all the concentrations of all materials go down, but their rankings remain stable, probably with the exception that the rankings of some of the water handling proteins may change. In the future, a group of internal controls will be used, and the relative quantification of biomarkers compared to the internal control will be used as a biomarker. To make it simple, urine creatinine was used to normalize the volume in many studies.

Many of the previous studies used the counterparts' urine for comparison with mainly sex and age consideration. Different from blood, urine is supersensitive to many factors such as pointed out earlier in this chapter. Lifestyle differences, medication differences, and even geographic differences all cause urine variations. It seems almost impossible to have a perfect control group especially when there is only limited number of subjects that can be analyzed. It may only work when the two groups we plan to compare have very big differences, much bigger than the most confounding factors. Theoretically if we compare urine samples with one collected previously from the same person, assume there is no significant lifestyle change, the changes in urine should reflect the physiological or pathophysiological changes during this period of time. I propose that even for healthy people, urine should be collected and stored as control for comparison with the sample at the next time point (Gao 2017).

In this case, a huge number of samples will be saved since every person should save one urine sample once in a while. The cost will be huge if we put them all in −80 freezer. After analyzing where the cost comes from, we realized that the information in urine is from the dry part of the urine, but saving the water part is expensive. To solve the problem, water should be removed before storage. Using membranes with affinity to a group of macromolecules such as nitrocellulose for proteins, we can extract proteins out of urine onto the nitrocellulose. The nitrocellulose membrane with proteins on it, we call it urimem, can then be dried and sealed in a vacuum bag. This method can be so cheap; a large number of clinical samples can be stored even for healthy people for future prospective studies (Jia et al. 2014). To do it in large scale, automation and standardization are inevitable, not only the automation of making urimem but also automation of urine analysis. Urine should be automatically processed and urinary proteins loaded into the LC-MS/MS system in clinical laboratory. A huge amount of urinary information can then be generated and related to the corresponding biological process. This may only be done when the potential of urine as an early biomarker source is realized and recognized by scientists, by investors, and by governments.

Urine can be collected completely noninvasively. But it is always considered as part of a human being ethically. Should we have a separate category for human wastes? For this category as long as the waste sample is deidentified, it has no harm to the donor completely. The donation of this type of waste samples should be mandatory for participants of all national health programs. The samples and related deidentified information should be available free to nonprofit researchers for the benefit of human kind (Gao 2015).

Urine may not be only used as biomarker source for the purpose of stopping or curing diseases. It can be the information resource for understanding mechanisms of known or even unknown diseases. From existing studies, many differential urinary proteins in animal models have shown the relation with the disease mechanisms. In case of a new disease, differential urinary proteins may point to the systems, the functions, and/or pathology of organs the disease interferes.

With the better understanding of the mechanisms of the disease, we will have a clearer direction for drug discovery and disease management.

Animal models are very useful tools for understanding disease mechanisms and finding potential biomarkers. But not all disease has good model. From the system point of view, drug intervention may be a way to look for clues to the disease. Drugs may either remove the cause or reduce the effects or increase the resistance. Of course, drugs also have unwanted effects too. Careful analysis of differential urinary proteins of many different effective drugs with different side effects may help to remove the effects of unwanted effects of each drug and provide the clue of the disease-related pathways. This is only a theory, an alternative way for studying diseases without a good model. How effective it can be remains to be seen (Gao 2018).

A small obstacle still worth mentioning is the publication of studies in urinary biomarker field. There are not many laboratories working in this field. Many people outside of this field may not understand and not be as optimistic. There are very few funding supporting studies in this field. But the reviewers are all critical. They want to see everything starting from the discovery, the mechanisms to the validation even to the clinical use at the bedside. It is not realistic now with limited funding for this small group of researchers. In the deep mind of the reviewers, it buried the doubt and disbelief. In my humble opinion, any paper providing an interesting clue is worth publishing. Free academic atmosphere nurtures big innovation. Scientists as reviewers in this field should understand the situation and support each other to make this field known which eventually benefits all. A post-publication review procedure may help to promote a neglected field. A candidate drug effective in animal model for a major disease can make big news in major journals and public media. But a panel of candidate urinary biomarker for a major disease, which may potentially make early diagnosis and change the course of the disease, cannot make even a ripple in the scientific community. How fast this field grows will determine how early we can benefit from it. There was no journal name containing the word "urine" in thousands of scientific journals until <URINE> was launched by visionary KeAi publishing. Supporting a journal focusing on this field will benefit the field and every party in the field, and eventually the human being.

Read on. Era of urine is coming.

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Chapter 2
Human Urine Proteome: A Powerful Source for Clinical Research

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Abstract As noninvasive and easily available biological fluid, urine is becoming an ideal sample for clinical disease biomarker study. In recent years, researchers endeavored in profiling urinary proteome and discovering potential disease biomarkers. However, there are still many challenges in the studies of urinary proteome due to the complexity of urine. In this article, we review current status of urinary sample preparation, including collection, storage, and extraction of urinary proteins, and the overall urinary proteome analysis so far, which may be helpful for urinary proteome analysis.

Keywords Urinary proteome · Mass spectrometry · Preparation and storage

2.1 Introduction

Body fluids have been regarded as the significant source of biomarkers, which could be used for the early diagnosis and state forecast of disease in clinical (Hu et al. 2006; Elkind et al. 2006; Rossing et al. 2005; Cicenas et al. 2005). As a body fluid, urine is an important resource of disease biomarker discovery.

Urine is excreted by the kidney to eliminate waste products from plasma. About 150–180 l of plasma is filtered by glomeruli to develop the "primitive urine." More than 99% of "primitive urine" is reabsorbed by the renal tubule and the "final urine" is remained to be excreted (Decramer et al. 2008). Approximately 30% of urinary proteins originate from the plasma proteins, whereas 70% comes from the kidney and the urinary tract (Thongboonkerd et al. 2002a). Therefore the urinary proteome might supply important biomarkers directly reflecting the functions of the kidney and related organs (Wu et al. 2010). As one of the most attractive sources for biomarker detecting, urine has showed several advantages: (1) urine can accumulate changes from the body: most of the waste in the blood that reaches the urine can tolerate a much higher degree of change; thus, biomarkers in urine are more likely

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to be magnified and detectable than in blood (Gao 2013); (2) it is easy to be collected in large amount and noninvasive way; (3) urine samples are less complex than plasma and carry many proteins, peptides, and amino acids that have not been discovered in plasma (Anderson et al. 1979a). Therefore, many researchers did their best to have a deeper understanding of urine proteins and discover potential biomarkers in recent years.

However, there still are many difficulties and problems needed to further explore and study. In this review, we summarize achievements of urinary proteomics, including sample collection, preparation, and urine proteome analysis in recent years, which may be helpful for further studies.

2.2 Collection and Storage

2.2.1 The Types of Urine

In clinic, many kinds of urine samples have been used according to the different examination. However, many factors may influence the components of urinary proteome (Sun et al. 2009), including daily activities, physiological variations, and environmental factors such as temperature and pH. Therefore, several types of urine samples have been analyzed by proteomic approach, including first morning urine, 24-h urine, second morning urine, random urine, the urine collected after drinking a large amount of water or after drinking coffee, etc.

24-h urine can show the excretion of urinary proteins within a day (Thongboonkerd 2007). But the collection of 24-h urine depends on patient compliance, which is unpractical to be completed entirely and easy to have some errors during the collection process (Bottini et al. 2002). Concerning first morning urine, it can't exhibit urine "diurnal variation" (different time-points' variation in all day) (Thongboonkerd 2007). And Hoorn et al. (2005) reported that first morning urine may have bacterial contamination due to the long residence time in the bladder.

Sun et al. (2009) made a qualitative and quantitative analysis of five samples (first morning void, second morning void, excessive water drinking void, random void, and 24 h void) collected in 1 day from healthy volunteers by 1-D LC/MS. They found no significant differences in the protein numbers of these five samples, and 42 common proteins to five samples contributed an average of 88.7% of abundance to each sample. Other studies (Thongboonkerd et al. 2006; Peerapen et al. 2017) compared four different time-point urine, including first morning urine, afternoon urine, water loading urine, and urine after drinking a cup of coffee. They found the first morning urine contained greater amount of proteins but less protein spots in 2D gel than afternoon urine. The water loading urine had the least amount of proteins by 2D-PAGE analysis but exhibited a few newly presenting spots. There were more spots in the sample after caffeine ingestion than in water loading urine.

To avoid bacterial contamination deriving from skin contamination, midstream urine is usually recommended as the standard for urinary proteome analysis, par-

ticularly for women (Lifshitz and Kramer 2000). Schaub et al. (2004) employed surface-enhanced laser-desorption/ionization time-of-flight mass spectrometry (SELDI-TOF-MS) in profiling the first-stream urine and midstream urine from three females and three males. For male samples, there were no observable differences between midstream and first-void. But for female samples, first-void urine emerged three specific SELDI peptide peaks after a 3-day storage compared with midstream.

2.2.2 Protease Inhibitor

Protease inhibitors had been initially suggested to be used to prevent proteolysis of clinical biological fluids, which was caused by endogenous proteases (Havanapan and Thongboonkerd 2009). However, whether protease inhibitors are necessary for the studies of urinary proteomics is debatable. Shinada et al. (2000) incubated 30 kDa nonglycosylated [^{125}I] IGFBP-3 with urine samples and found IGFBP-3 proteolysis by SDS-PAGE analysis. But Havanapan and Thongboonkerd (2009) studied the effect of protease inhibitors cocktail on midstream random urine specimens and found that no observable qualitative and quantitative changes by two-dimensional gel electrophoresis (2-DE) analysis. Thongboonkerd (2007) suggested protease inhibitors were unnecessary for the studies of nonproteinuric urine because there were lower amounts of proteases in urine than in plasma, cells, or tissues. In 2010, Maryam Afkarian et al. demonstrated that absence of protease inhibitors did not affect the identification of the high confidence proteins (Afkarian et al. 2010). In the same year, Kania et al. (2010) investigated the albumin fragmentation by using the method of nephelometry, HPLC, and sodium dodecyl sulfate-polyacrylamide gel electrophoresis. Albumin concentration measured by HPLC was most dramatically affected, with near-complete loss of albumin-sized material within 1 h of incubation at pH 2.3–2.5. Their results showed that urinary albumin digestion occurred in a manner consistent with the activity of endogenous urinary proteases. And they recommended that the adjustment to neutral pH or addition of protease inhibitors may be useful techniques for urine sample preservation.

2.2.3 Preservatives

During the storage, urine samples might have bacterial overgrowth, which could change the urinary proteome (Thongboonkerd and Saetun 2007). Therefore preservatives were recommended to prevent the bacterial overgrowth after collection (Thongboonkerd and Saetun 2007). Thongboonkerd and Saetun (2007) studied the effects of the addition of either sodium azide (NaN_3) or boric acid on bacterial overgrowth in pooled urine from five healthy individuals. They found the addition of NaN_3 and boric acid could delay the bacterial overgrowth, and greater delay (for at

least 48 h) was obtained by relatively higher preservatives. They recommended addition of 2–20 mM boric acid or 0.1–1 mM NaN_3 to one void random urine specimens and addition of 200 mM boric acid or 10 mM NaN3 to 24-h urine collection.

Recently, Remer et al. (2014) examined the long-term stability and validity of analyte concentrations of 21 clinical biochemistry parameters (creatinine, urea, iodine, nitrogen, sodium, uric acid, and so on) in 24-h urine samples stored at −22 °C and preservative-free. They suggested high long-term (>10 years) stability and measurement validity for numerous clinical chemistry parameters when stored at −22 °C without any urine preservative. Porter IA et al. (Porter and Brodie 1969) reported that boric acid adequately could preserve urine specimens for up to 24 h. They compared the results of bacteriological culture and microscopic examination of urine samples transported over a distance by the dip-inoculum transport medium, icebox, and boric acid preservation with "natural" urine specimens, and the results showed that the "natural" urine gives satisfactory preservation.

2.2.4 Storage Temperature

Appropriate storage temperature could decrease degradation of urine proteins to some extent. In 1999, Klasen et al. (1999) analyzed changes of albumin concentrations after urine samples were stored at 4 °C, −20 °C, and − 70 °C. They found that if samples could be analyzed in 4 weeks after collected, the best storage temperature was 4 °C. And they recommended −70 °C is used as storage temperature for longer storage. They did not suggest storage temperature − 20 °C because they found the IgG concentrations decreased after 1-week storage at −20 °C. In 2007, Thongboonkerd and Saetun (2007) reported that to prevent the bacterial overgrowth, uncentrifuged urine samples without preservatives should be kept no longer than 20 h at 4 °C. Without any preservative, urine samples should not be stored at room temperature for longer than 8 h. In 2008, Lee et al. (2008a) reported that peptide and urine proteins are very stable at room temperature up to 24 h by SDS-PAGE analysis. In 2011, Molina et al. (2011) evaluated the impact of temperature after urine collection and before freezing at −80 °C. In their study, urines were kept at room temperature (RT) for 1 h and then stored at +4 °C or at RT for another 7 h, and no quantitative difference was found by 2D-GE. But Hindman et al. (1976) reported that urine stored at room temperature for more than 2 h showed an overgrowth of microorganisms.

2.2.5 Freeze-Thaw Cycle

Previous studies showed freeze-thaw cycles could influence the components of body fluid proteome, including serum/plasma and cerebrospinal fluid (Rosenling et al. 2009; Hsieh et al. 2006). Studies also showed that when urine samples were stored at low temperature, researchers should avoid freeze-thaw cycles

(Thongboonkerd 2007). Schaub et al. (2004) analyzed first-void and midstream urine specimens from three females and three males by SELDI-TOF MS. Results indicated that after 1 to 4 freeze-thaw cycles, the urinary proteome did not change remarkably except for the loss of intensity in some peaks, whereas some small peaks were undetectable after the fifth freeze-thaw cycle. Besides, some studies (Lee et al. 2008a; Powell et al. 2006; Bao 2009) reported the degradations of some proteins resulted from 4 to 7 freeze-thaw cycles in urinary proteomics. Furthermore, Klasen et al. (1999) found some proteins forming precipitates after storage and thawing. According to the studies of Saetun et al. (2009), after overnight storage at −20 °C, urinary proteins may precipitate, and they found that EDTA (5 mM) could reduce the amount of precipitates and pH could influence the type of precipitates. To redissolve the precipitates, effectively shaking of the specimens should be done at room temperature.

2.2.6 pH

Thongboonkerd et al. (2009) analyzed pH adjusted urine samples which were precipitated by 75% ethanol. The 2-DE results showed that different pH levels did not influence the consistency of individual urine specimens and the total number of spots. Therefore, they thought it was unnecessary to adjust the pH of urine samples before gel-based proteome analysis.

2.2.7 Standard Protocol for Urine Collection

Based on previous studies, a standard protocol for urine collection was recommended by Human Kidney and Urine Proteome Project, HKUPP, and European Urine and Kidney Proteomics, EuroKUP, Initiatives from December 9, 2009.

The details were described as follows:

Standard Protocol for Urine Collection (http://www.hkupp.org and http://www.Eurokup.org)

1. Type of urine sample

Midstream of second morning urine (preferably) or morning random-catch urine, in sterile (preferably) or clean urine collectors.

2. Pretreatment and storage

Centrifuge at 1000 g for 10 min to remove cell debris and casts. Aliquot supernatant avoiding disturbing the pellets at 1.5, 10, or 50 ml (depending on downstream application). Do not overfill the tubes. Store at −80 °C (preferably) or − 20 °C. Record time until freezing (it should be no longer than 3 h).

3. Freezing and thawing

Avoid freeze-thaw cycles. If thawing and re-freezing occurs, always keep a record of this event.

Notes: http://www.hkupp.org and http://www.Eurokup.org.

2.3 Urine Preserved on Membrane

In 2014, Jia et al. (2014) proposed a method for adsorbing urinary proteins onto a polyvinylidene difluoride (PVDF) membrane named Urimem. In their study, urine samples were filtered through the membrane, and urinary proteins were adsorbed on it. The proteins on the membrane were dried and could be stored in a vacuum bag to keep the preservation of protein pattern faithfully. The loading capacity of the PVDF membrane was tested using SDS-PAGE analysis. The results showed that most of the proteins were adsorbed on the membrane during the first adsorption. The method has showed several advantages due to simplicity, low cost, nonrequirement of organic solvents, and minimal sample handling. Furthermore, proteins on the membrane could be even stored at room temperature for more than 2 weeks.

Based on Jia's work, similar as Jia et al. (2014), Somchai Chutipongtanate et al. (2015) developed a simple rapid method of urine preparation named syringe-push membrane absorption (SPMA) in 2015, which combined 5-mL medical syringe with protein-absorbable membrane. In the study, they found nitrocellulose to be the most suitable membrane to combine with SPMA, which provided the greatest quality of proteome profile by 2-DE analysis. Comparing with three current methods of urine preparation (i.e., ultrafiltration, dialysis/lyophilization, and precipitation), nitrocellulose-SPMA had better working performance due to acceptable recovery yield, less workload, short working time, high accessibility, and low unit cost. In addition, protein absorbed on nitrocellulose harvested from the SPMA procedure could be stored as a dried membrane at room temperature for at least 1 month without protein degradation or modification.

For the protein recovery from the membrane, Qin and Gao (2015) developed a method to elute the urinary proteins from nitrocellulose membrane with heating. They raised the temperature to reduce the intense vertexing time, and gentle rotating was kept while precipitation to prevent nitrocellulose reformation. By SDS-PAGE and LC-MS/MS, the urinary proteins prepared by heating elution procedure showed no degradation of proteins.

2.4 Urine Preparation

For urinary proteomics, crude urine samples are complex including high concentrations of salts, small molecules, and some metabolic wastes (Tantipaiboonwong et al. 2005a), and concentration of urinary proteins are too low to be identified. So

many sample preparation methods have been applied to concentrate urine proteins and remove small molecules, such as organic solvent precipitation, ultracentrifugation, dialysis-lyophilization, and ultrafiltration (centrifugal filtration) (Tantipaiboonwong et al. 2005a; Khan and Packer 2006).

2.4.1 Organic Solvent Precipitation

Organic solvent precipitation is a popular method in urinary proteomics. Organic solvent can reduce permittivity of urine and break hydration shell on the surface of protein molecules, thus urine proteins gathered and are precipitated effectively. Organic solvent precipitation method had several characters. First, this approach can be used to handle larger volumes of urine and takes less time than other methods (Khan and Packer 2006). Second, it can enrich higher molecular weight protein species than ultrafiltration (Saetun et al. 2009). Third, it can effectively precipitate more acidic and hydrophilic proteins than ultracentrifugation (Thongboonkerd et al. 2002b).

Studies showed types and concentration of organic solvents may play various roles on urinary protein precipitation (Lifshitz and Kramer 2000; Khan and Packer 2006). Khan and Packer (2006) employed 2-DE to analyze the urinary proteome precipitated by different organic solvents and found that higher resolution and more protein spots could be obtained using acetonitrile (urine-to-solvent ratio was 1:5). Tantipaiboonwong et al. (2005a) mentioned that addition of trichloroacetic acid as well as trifluoro acid could increase protein yield. Thongboonkerd et al. (2006) made a comprehensive comparison of different organic solvent precipitation methods by 2-DE. They revealed applying 90% or 75% organic compounds could get greater recovery yield than lower percentage of organic compounds. Ethanol, methanol, or acetone precipitation methods were suggested to obtain more protein spots and higher protein recovery yield in routine or gel-based urinary proteome studies. Moreover, acetonitrile precipitation was suggested for proteinuric urine or a larger volume of urine for its highest number of protein spots but lower protein recovery yield. Besides, Simpson and Beynon (Simpson and Beynon 2010) found that acetone precipitation may lead to selective modification of peptides, predominantly in the peptides whose second amino acid is glycine residue, which might generate a relatively stable derivative. However, Maryam Afkarian et al. (2010) reported that protein extraction by methanol precipitation would lead to the highest protein yields and the most reproducible spectra.

According to previous studies, disease state and the physicochemical property of urine sample would also affect protein extraction by different solvents (Olszowy and Buszewski 2014). In 2013, Crowell et al. (2013) provided an in-depth characterization of protein recovery through acetone precipitation. They increased the ionic strength of the solution by adding 1–30 Mm NaCl into acetone (50–80%), which dramatically improved the precipitation efficiency of individual proteins, and proteome mixtures (ca. 80–100% yield).

2.4.2 Ultracentrifugation

Ultracentrifugation is a common method for separating proteins due to easy sedimentation of high-density protein molecular under ultracentrifugation situation. In 2002, Thongboonkerd et al. (2002b) analyzed urines from five normal donors by 2-DE and matrix-assisted laser desorption ionization time-of-flight (MALDI-TOF). They found ultracentrifugation method could fractionate more basic, hydrophobic, and membrane proteins than organic solvent precipitation approach. But ultracentrifugation uses expensive equipment and acidic proteins are lost, which might limit its application (Thongboonkerd et al. 2002b).

2.4.3 Dialysis

Another commonly used method is to combine dialysis with lyophilization (Oh et al. 2004). Oh et al. (2004) reported that dialysis-lyophilization approach was likely to profile the whole urine proteins on 2-DE and could improve reproducibility and resolution, because it could effectively remove the molecules that interfered the profiling of 2-DE. Moreover, Thongboonkerd et al. (2006) revealed that this method had great protein recovery yield but showed lowest number of protein spots compared with precipitation, ultracentrifugation, and ultrafiltration method.

2.4.4 Ultrafiltration

As for ultrafiltration method, it uses ultrafiltration membranes to discard small molecules and concentrate urinary protein according to molecular weight difference. Court et al. (2011) reported that ultrafiltration method enriched lower molecular weight proteins than 6% TCA precipitation by SDS-PAGE gel. Based on ultrafiltration method, Vaezzadeh et al. (2010) put forward a one-step sample preparation method. They added urine sample together with anti-HSA resin to a Vivaspin 6 spin-filter, which could isolate proteins and remove human serum albumin in one step. It realized sample concentration, purification, and albumin depletion simultaneously. Furthermore, they found that neutral pH (7–8) could achieve both efficient depletion and high protein recovery. In 2005, Payungsak Tantipaiboonwong et al. (2005b) reported that the sequential preparation of urinary proteins by gel filtration and ultrafiltration obtained the highest number of protein spots on 2D gels and retained the most urinary proteins.

Recently Sebastian T. Berger et al. (2015) described a 96-well plate compatible membrane-based proteomic sample processing method. In their study, a large-pore hydrophobic PVDF membrane was used to efficiently adsorb proteins, resulting in fast liquid transfer through the membrane and significantly reduced sample process-

ing times. Finally, they identified 819 proteins in only 150 μL urine sample by using a 1 h gradient on TripleTOF 5600 + .This method not only was high-throughput and very fast but also could prepare peptide samples by using urine sample directly without protein extraction. Besides, Yanbao Yu et al. (2017) described a FASP method adapted to 96-well filter plates, named 96 FASP, which could also prepare peptide samples by using urine sample directly. In this method, ~10 μg of total urinary protein was reduced, alkylated, and digested in 96-well filter plates directly, resulting in 700–900 protein identification by Q Exactive. The method was suitable for high-throughput quantitative clinical proteomics.

2.5 Normal Human Urinary Proteomes Analysis

Many researchers had undertaken studies to catalog the normal human urinary proteome. The first study came from Anderson et al. (1979b) in 1979. They found 250 urine protein spots by 2-DE. But without high-throughput protein identification approach, it was hard to profile the components of urinary proteome. The development of two ionization methods in MS, matrix-assisted laser desorption ionization (MALDI) and electrospray ionization (ESI), made the precise analysis of biomacromolecule possible (Costello 1997). In 2001 Spahr et al. (2001) firstly employed LC-MS approach to analyze the human urinary proteome and identified 124 urinary proteins. After that many groups contributed their efforts to profile a comprehensive normal human urinary proteome. 2-DE and LC-MS were two popular approaches for proteome analysis.

2.5.1 2-DE Approach

In 2002 Thongboonkerd et al. (2002b) reported their study utilizing acetone precipitation and ultracentrifugation preparation methods. By 2-DE and MALDI-TOF, they identified 47 unique proteins, 28 from acetone-precipitation method and 19 from ultracentrifugation method.

In 2004, Oh et al. (2004) prepared urine samples by dialysis-lyophilization and removed albumin using Affi-Gel Blue. They identified 113 urinary proteins on 2-DE by peptide mass fingerprinting with MALDI-TOF-MS analysis. In the same year Pieper et al. (2004) reported a large-scale urinary proteome analysis. First, they fractionated urine proteins by size exclusion chromatography and collected two fractions, higher than 30 kDa and lower than 30 kDa. Then they employed immunoaffinity subtraction chromatography to remove albumin and immunoglobulin G from higher than 30 kDa fractions. At last the two fractions were separated by 2-DE. Total 1400 distinct protein spots were found and 420 spots of these were identified to 150 unique protein.

In 2005, Smith et al. (2005) collected 35 urine samples from 12 donators, extracted the urinary proteins by solid phase extraction method. By 2-DE and MALDI-TOF/TOF analysis, 48 nonredundant proteins were identified.

In 2006, Khan and Packer (2006) used ultrafiltration and different organic solvent precipitation method to isolate urinary proteins, and a total of 339 proteins were found with 2-DE separations followed by MALDI-TOF analysis. Zerefos et al. (2006) exploited preparative electrophoresis to separate urinary proteins by 2-DE and MALDI analysis; 778 protein spots were found and 141 proteins were identified.

2.5.2 LC-MS

In 2002, Pang et al. (2002) applied 2D LC-MS method and identified 51 urine proteins from normal human urine proteome.

In 2005, Sun et al. (2005) applied three approaches to analyze the urinary proteome, 1DE plus 1D LC-MS, direct 1D LC-MS, and 2D LC-MS. They identified 226 urinary proteins, 171 proteins of which were identified for the first time. Castagna et al. (2005) used hexameric peptide libraries methods to reduce the high abundant proteins and enrich medium and low abundant ones in urinary proteome. By this method they identified 383 unique proteins and 251 proteins were not ever found.

In 2008, Lee et al. (2008b) handled urine samples by four different approaches: vacuum centrifugation, 90% ethanol precipitation, microconcentrator, and reverse phase trapping column. By in-gel digestion and LC-MS analysis, 154, 154, 162, and 148 proteins were identified, respectively, in four preparation methods and 600 proteins were found in total (Marimuthu et al. 2011).

In 2009, Kim and Moon (2009) modified isoelectric focusing and asymmetrical flow field-flow fractionation by applying Teflon tubing to connect multilane asymmetrical flow field-flow fractionation (AF4) channel with isoelectric focusing (IEF) channel (prevent the possible protein adsorption by membrane wall of IEF). The fractions from IEF were analyzed by LC-MS and 245 urinary proteins were identified.

Urinary protein posttranslational modification was an important issue for urinary proteome analysis. In 2006, Wang et al. (2006) utilized concanavalin A to enrich N-linked glycoproteins from normal urinary proteome. By 1DE plus 1DLC-MS and 2DLC-MS, total 225 glycoproteins were identified, 150 annotated as glycoproteins by Swiss-Prot and 43 by NetNGlyc 1.0.

2.5.3 High-Resolution MS Analysis

Along with great improvement of mass accuracy of mass spectrometer, new generations of high-resolution MS dramatically increased protein identification for proteomics (Olsen et al. 2005).

In 2006, Jun Adachi et al. (2006) reported the first urinary proteome analysis by high-resolution MS. They analyzed in-gel and in-solution digestion urinary samples by LTQ-FTICR and LTQ-Orbitrap. By combining 1281 proteins from LTQ-FTICR with 1055 proteins from LTQ-Orbitrap, total 1543 urine proteins were obtained from this in-depth study. Gene ontology (GO) analysis showed that membrane proteins occupy nearly half of the annotated proteins. Extracellular proteins were overrepresented and intracellular proteins were underrepresented. However, plasma membrane proteins and lysosome proteins were unexpectedly overrepresented.

In 2010, Goo et al. (2010) analyzed the urine samples from ten female healthy persons by LC coupled with a hybrid linear ion trap-orbitrap mass spectrometer and identified 1003 urinary proteins. Li et al. (2010) used urines from three healthy male donors, and digested peptides were fractionated by two approaches, integrated multidimensional liquid chromatography and Yin-Yang multidimensional liquid chromatography methods. 6739 unique peptides and 1310 nonredundant proteins were obtained by two approaches. Furthermore, they did the first large-scale work to profile urinary phosphoproteome and found 45 unique phosphopeptides from 31 phosphoproteins. Most of the phosphorylation sites were on serine residues except for six on threonine and only one on tyrosine residues.

In 2011, Marimuthu et al. (2011) reported the first urinary proteome result of high-resolution MS/MS with LTQ-Orbitrap Velos mass spectrometer. They exploited in-gel digestion and LC-MS approach to analyze unfractionated proteins of the pooled urine, as well as the glycoproteins after the lectin affinity enrichment. 1452 proteins were found in unfractionated urine and 617 proteins in glycoproteome. Total 1823 proteins were found, and 671 proteins of these proteins were identified in human urine for the first time. 265 proteins out of 617 enriched proteins were glycosylated. Forty-four peptides out of 131 peptides identified with protein N-terminus were analyzed to be acetylated.

In 2013, Zheng et al. (2013) performed a proteomic analysis of urine samples from pregnant and nonpregnant patients using SDS-PAGE and LC-MS/MS. In total, 2579 proteins were identified, including 1408 from the urine of pregnant volunteers and 1985 from the nonpregnant group. Total 1023 proteins were not reported in previous studies.

In 2017, Zhao et al. (2018) presented an in-depth analysis of the urinary proteome based on different separation strategies, including direct 1D LC/MS/MS, 2D LC/MS/MS, and gel-eluted liquid fraction entrapment electrophoresis/liquid-phase isoelectric focusing followed by1D LC/MS/MS. By combining 799 proteins from 1D LC/MS/MS with 2362 proteins identified in 2D analysis and 2924 proteins from 3D analysis, total of 6085 proteins were identified in healthy urine, of which 2001 had not been reported previous. The protein functional analysis showed extracellular proteins and plasma membrane proteins were enriched in 1D analysis, proteins identified in 2D analysis were enriched in intracellular proteins, and proteins in 3D analysis were most in the cytoplasm and nucleus. Moreover, by mapping the urine protein to Human protein Atlas, the tissue distribution of normal urinary protein is also provided. The urinary proteome distributes across 44 tissues; among them, the brain is the tissue with the highest level of both protein and mRNA expression, and

other tissues with more highly expressed proteins were mostly digestive organs, such as the colon and stomach.

Recently Zhao et al. (2015) reported a comprehensive comparison of five body fluids, including urine, plasma, saliva, cerebrospinal fluid, and amniotic fluid using 2D LC/MS/MS approach.

A total of 4717 proteins were identified, and 564 proteins were shared among the five body fluids, with common functions in the coagulation/prothrombin system and inflammatory response. A total of 36.7% of the proteins were detected in only one body fluid and were closely related to their adjacent tissues by function. The functional analysis of the remaining 2986 proteins showed that similar functions might be shared among different body fluids, which highlighted intimate connection in the body. Above results indicated that body fluids might reflect the diverse functions of the whole body rather than the characteristics of their adjacent tissues.

2.6 Conclusion and Outlook

Following the development of MS technologies, precision proteomics become more and more significant in proteomics. It could not only reveal more proteins secreted in urine but also avoid more errors which lead to misdirected results (Mann and Kelleher 2008). Especially, in 2011 Marimuthu et al. (2011) published the first urinary proteome study with both of MS and MS/MS at high resolution exhibiting more credible results. With the application of urinary proteome to clinical researches, a larger precision urinary proteome database should be developed, which should be used as a reference for further study.

Another important issue for urinary proteome was high-throughput quantitation. Quantitation of urine proteins has been proposed, and many approaches were exploited to realize relative and absolute quantitation. In 2013, Nolen et al. (2013) applied multiplexed bead-based immunoassays and made absolute quantitation of 211 proteins in healthy urine samples. However, more than 600 proteins could be identified in only one 1DLC-MS run (Nagaraj and Mann 2011). Therefore, high-throughput urinary protein quantitation, especially absolute quantitation, still needs more concern.

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Conflict of Interest The authors have declared no conflict of interest.

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Chapter 3
Comparison of Urinary Proteomes Among Three Animal Models

Na Ni and Jianqiang Wu

Abstract Biomarkers are the monitorable changes associated with physiological or pathological changes. Urine is not regulated by the homeostatic mechanism and can reflect multiple changes in the body. Animal models can simulate human disease processes, monitor disease changes, and provide clues for early diagnosis. In this chapter, clues are provided for the dominant model animals associated with disease selection by comparing the urine proteome of rats, guinea pigs, and golden hamsters. The peptides were cleaved by membrane digestion and analyzed by LC-MS/MS. The number of urine proteins in the three different animals was different, and also different in every system of the body. This provides a basis for selecting the best animal models for different diseases.

Keywords Urine proteomics · LC-MS/MS · Animal model · Rat · Guinea pig · Golden hamster

3.1 Introduction

The most important finding for biomarkers is to look for detectable changes associated with physiological and pathophysiological processes (Gao 2013). Urine is not regulated by the homeostatic mechanism and can reflect changes in metabolism in the body, making it easy to collect. Therefore, urine is a good biological source for finding disease markers (Gao 2015).

However, human clinical urine samples are affected by a variety of physiological or pathological effects, such as age, gender, diet, exercise, and drugs (Wu and Gao 2015). Animal models are the most effective way to find the causal relationship

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between diseases for the following reasons: (1) using animal models to reduce the effects of genetic and environmental factors on urine proteomics; (2) helping to identify biomarkers at each stage, including early diagnosis; (3) because clinical patient care is unavoidable, animal models can avoid the effects of drugs on the proteome. Therefore, the use of animal models for the study of urine protein markers is more economical and controllable, which is conducive to early observation of diseases and monitoring of related pathophysiological changes during disease progression (Zhao et al. 2014).

In research activities, rats are commonly used to establish disease animal models and conduct proteomic research, such as rat kidney disease model (Carter et al. 2016; Rosner 2009), rat coronary artery disease model (Paapstel et al. 2016; Zimmerli et al. 2008; Mullen et al. 2011), and rat bladder cancer model (Zhu et al. 2016; Vrooman and Witjes 2008). However, rats are not the dominant model animals for all diseases. Different diseases should choose their corresponding dominant model animals. Therefore, it is more necessary to study the superior models and proteomes of other animals. The hamsters commonly used as experimental animals are mainly the golden hamster and the Chinese hamster. The golden hamster has 38 inbred lines, 17 mutant lines, and 38 distant crosses. The hamster is mainly used in biomedical research in oncology research, reproductive physiology and family planning research, vascular physiology and microcirculation research, nutrition research, and infectious disease research. The hamster is the main biological material for the study of rabies virus and Japanese encephalitis virus and its vaccine production (Cui et al. 2014). Guinea pigs, also known as the squirrel, are widely used in immunology, nutrition, physiology, toxicology, and infectious disease research.

In this chapter, we analyze the difference in urinary proteins between rats, golden hamsters, and guinea pigs to provide basic data for medical experiments and provide a basis for the selection of dominant animal models.

3.2 Results and Analysis

Urine proteins were precipitated with ethanol and resuspended in lysis buffer. Urine proteins were digested with trypsin using the FASP method. Peptide mixtures were desalted and analyzed with LC-MS/MS using ABSCIEX Triple-TOF5600 mass spectrometer. The urinary protein of rat, golden hamster, and guinea pig was compared by SDS-PAGE. The differences were as follows: the rats differed significantly from others, while the golden hamsters and guinea pigs were more similar. The rat urinary proteins were mainly concentrated in 35–15 kDa, while the expressions between 35–25 kDa of guinea pig and golden hamster urinary proteins were weak (Fig. 3.1).

The results of urinary protein identification in rats, golden hamsters, and guinea pigs were searched by Mascot and then compared with human homologous proteins by UniProt and converted into adult homologous proteins. The comparison of three

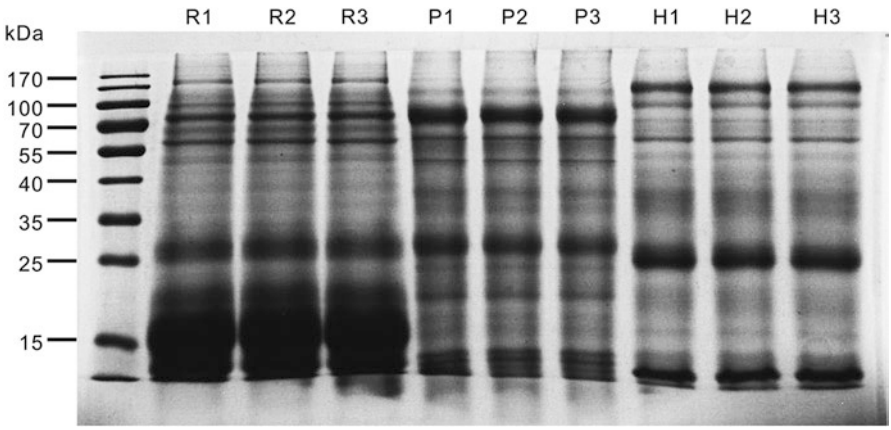

Fig. 3.1 SDS-PAGE analysis of urinary protein sample: R1-3, samples from rats; P1-3, samples from guinea pigs; H1-3, samples from golden hamsters. (Ni et al. 2018)

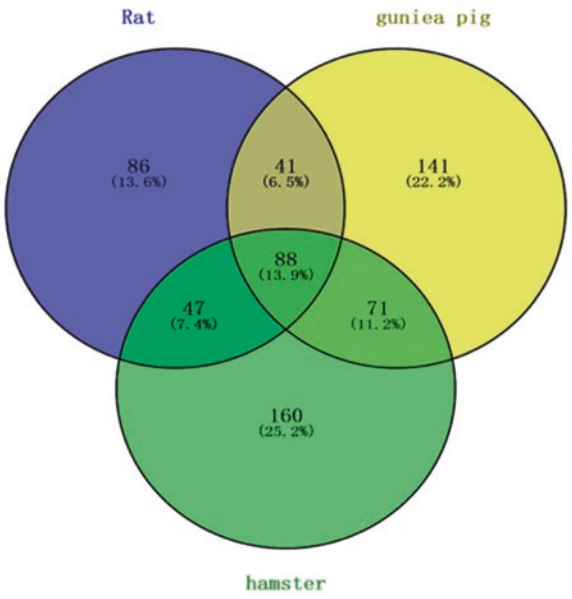

Fig. 3.2 Rat, guinea pig, and golden hamster urine protein comparison Venn diagram. The purple part represents 86 proteins that are unique to rats, the yellow part represents 141 proteins that are unique to guinea pigs, and the green part represents 160 proteins that are unique to golden hamsters. (Ni et al. 2018)

animal-human homologous proteins showed that there were 340 human homologous proteins in the urine of guinea pigs, 266 human homologous proteins in rat urine, and 366 human homologous proteins in the urine of golden hamsters. As shown in Fig. 3.2, there are 88 trusted proteins in all three species, while 86 proteins are unique to rats, 141 proteins are unique to guinea pigs, and 160 proteins are unique to golden hamsters. As shown in Fig. 3.3, the similarity of guinea pig and golden hamster urinary protein is greater.

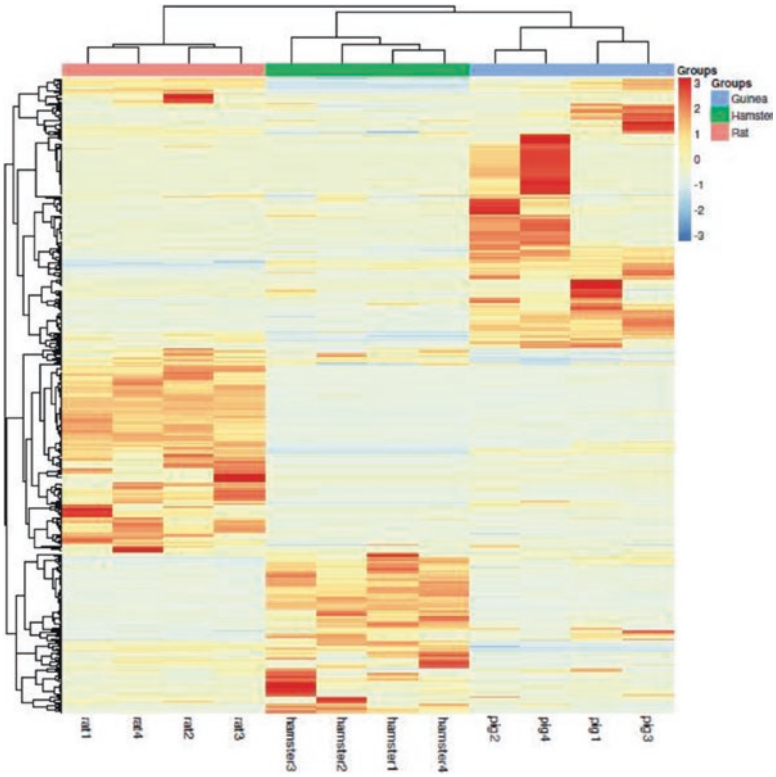

Fig. 3.3 The heat map of three kinds of animal urine protein. From left to right are: rats, golden hamsters, and guinea pigs. The expression of protein in golden hamster and guinea pig is close. (Ni et al. 2018)

All proteins identified in the urine of rats, golden hamsters, and guinea pigs were analyzed by PANTHER database, and the urine proteins of rats, golden hamsters, and guinea pigs were compared in terms of molecular function, biological processes, cellular components, and pathways (Fig. 3.4). The analysis showed that the urinary proteins involved in the three species were different in biological processes and pathways. They had different molecular functions and different cellular components. For example, guinea pigs and golden hamsters have more urinary proteins involved in the calcium signaling pathway, the integrin signaling pathway, and the Wnt signaling pathway than in rats.

The urinary proteins of rat, guinea pig, and golden hamster were compared with the Human Protein Atlas database. The results showed that the protein expression of the three species were different in the urinary system, digestive system, nervous system, respiratory system, immune system, and endocrine system, and the expression of urinary protein in golden hamsters is much higher than others. The expression of urinary protein in rats is the lowest (Fig. 3.5).

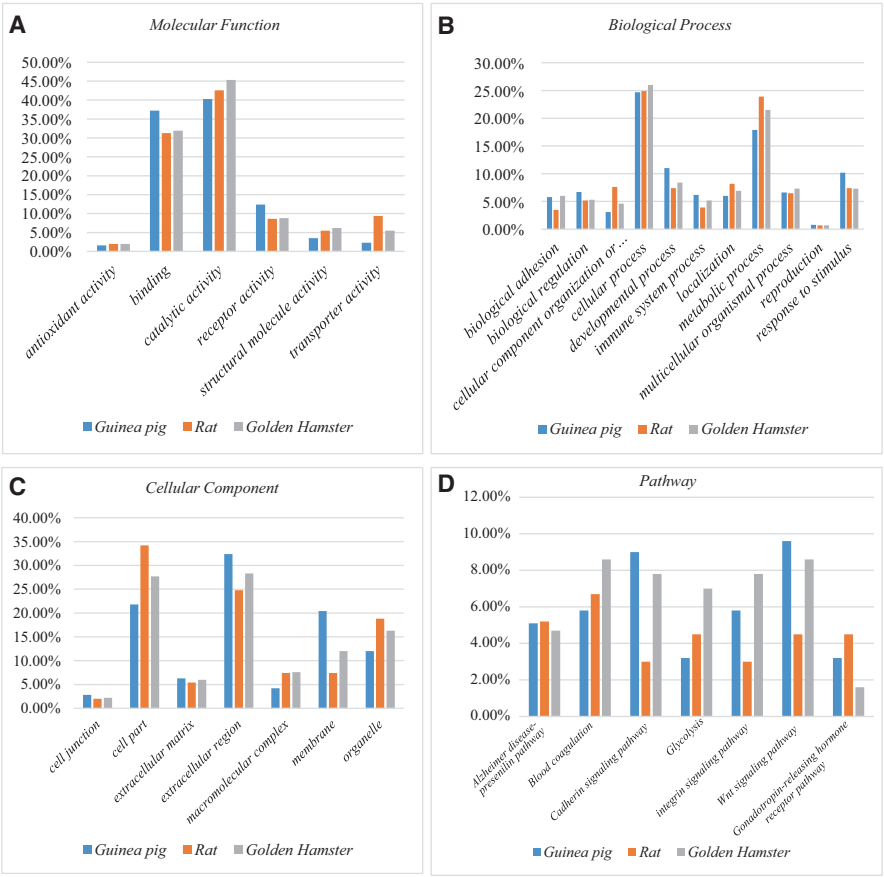

Fig. 3.4 Gene ontology analysis. There were significant differences in the expression of three animal proteins. (**a**) Molecular function. (**b**) Biological process. (**c**) Cellular component. (**d**) Pathway. (Ni et al. 2018)

3.3 Research Prospects

At this stage, rats are commonly model animals, and reports of studies using guinea pigs and golden hamsters are rare. This study compared the urine proteins of rats, golden hamsters, and guinea pigs by proteomic methods in order to lay the data foundation for medical experiments and provide clues for the selection of dominant animal models.

In this study, the urine proteins of rats, guinea pigs, and golden hamsters were found to be very different. It can be seen from SDS-PAGE that the three kinds of animal have different high-abundance proteins and different abundance inhibition, and the protein expression of guinea pigs and golden hamsters is relatively similar. Therefore, the use of rats alone to establish disease animal models is not suitable for

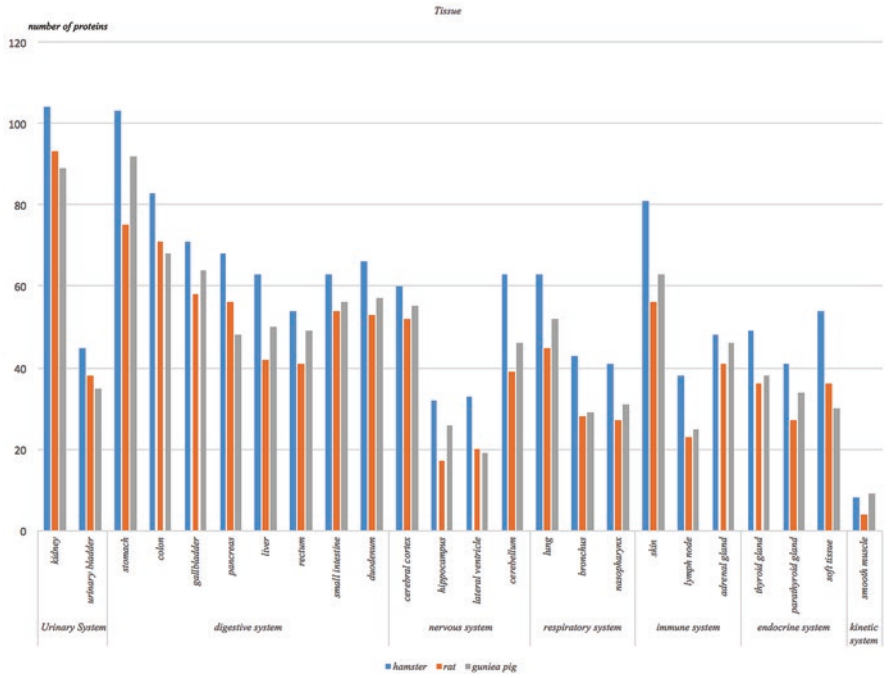

Fig. 3.5 The Human Protein Atlas result between rats, guinea pigs, and golden hamsters. In different organs, the urine protein expression of three animals was significantly different. Among them, the highest expression is of golden hamsters, followed by guinea pigs, and finally rats. (Ni et al. 2018)

studying all diseases. The expression of urinary protein in rats, guinea pigs, and golden hamsters was different in each organ of every system, and the biological processes involved in them were also different. The expression of rats was the least. It indicated that golden hamsters and guinea pigs are relatively more dominant in the establishment of human disease models. For example, in our data, it can be seen that in the liver and pancreas, the expression of urinary protein in golden hamsters is high. Now in the medical field, the mortality of intrahepatic cholangiocarcinoma and pancreatic cancer is high, the early diagnosis is difficult, and the prognosis is poor. It is particularly important to look for urine protein markers for both diseases. By consulting the relevant literature, it has been reported that golden hamsters are dominant model animals for intrahepatic cholangiocarcinoma and pancreatic cancer (Kawaura et al. 2011; Takahashi et al. 2011). In the skin and lungs, although the urinary protein expression of guinea pigs is not as high as that of golden hamsters, the urine of golden hamsters is less and thicker. On the urine samples, guinea pigs are more suitable. The early diagnosis and differential diagnosis of skin allergies, skin blemishes, and chronic obstruction pulmonary disease are difficult, and the treatment is poor. Therefore, we can find the urine protein markers for differential diagnosis and monitor the disease progression and disease efficacy. It has been

reported that the guinea pig is the dominant model of chronic obstructive pulmonary disease, skin irritation, skin dermatophytes, and other diseases (Basketter 2016; Cambier et al. 2017; Ramírez-Ramírez et al. 2017). Through the above research, the selection of disease animal models can be guided. For example, in the selection of liver and pancreatic disease models, we can give priority to golden hamsters. In disease models such as skin diseases, we can give priority to guinea pigs and combine the disease and animal characteristics and previous reports to correctly select the dominant model.

In summary, the use of guinea pigs, golden hamsters, and rats to select their corresponding disease models can improve the efficiency and accuracy of urine protein marker screening, and have more prospect in the search for urine protein markers for early diagnosis of human diseases.

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Chapter 4
Urimem, a Membrane that Stores Urinary Components for Large-Scale Biomarker Study

Lulu Jia, Fanshuang Zhang, and Weiwei Qin

Abstract Urine accumulates traces of changes that occur in the body and can be a promising sample for dynamic health monitoring. Research on the translation of urinary biomarkers is underway. However, the current methods of urine preservation limit the translation of urinary biomarkers from the laboratory studies to the clinical application. Here, we propose a method to adsorb urinary components onto a membrane named urimem. Urine samples were firstly filtered through the membrane, and then the urinary components were concentrated, dried onto membranes, and stored in a vacuum bag. This method is simple and inexpensive, requires minimal sample handling, uses no organic solvents, and is environmentally friendly. Based on this preservation method and noninvasive collection, urine may become one of the most promising samples to be "recorded" in medical history. And this method will promote the clinical transformation of urinary biomarkers.

Keywords Urinary proteins · Urinary microRNA · Biological sample preservation · NC/nylon membrane

4.1 Introduction

Urine is an ideal source for early and sensitive biomarker discovery. It can be obtained in large quantities using noninvasive procedures. Unlike blood which typically maintains a homeostatic internal environment, urine tends to reflect changes

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occurring inside the body. This property potentially makes urine a better biomarker source than blood (Gao 2013). Hundreds of candidate biomarkers associated with kinds of diseases have been reported in urine, including even the biomarkers derived from the brain, an organ anatomically away from urine (Harpole et al. 2016).

Because of its noninvasive collection and its potential to be biomarkers, urine is a promising sample for dynamic health monitoring. Research on the translation of urinary biomarkers is underway. Clinical validation of urinary biomarkers requires the preservation of large amounts of urine samples. However, the current methods of urine preservation limit the translation of urinary biomarkers from the laboratory studies to the clinical application. For example, cryopreservation, the primary preservation method, requires a significant amount of space due to large volume and low concentration of urine. Additionally, it cannot absolutely prevent the degradation of urinary components, such as proteins and microRNA, and the required cold chain during sample transportation is challenging and costly.

Simple and inexpensive urinary components' preservation method can be the starting point for long-term comprehensive urine sample storage and hence facilitates the translational research on urinary biomarkers. Here, we introduce a urimem method that adsorbs proteins or microRNA of the urine onto a membrane, and the membrane was dried and stored in a vacuum bag (Jia et al. 2014; Zhang et al. 2015). This method is economical and occupying small storage space. In addition, dry preservation state prevents the urinary components' degradation, thus making it possible for long-term comprehensive storage of urine samples.

4.2 Materials and Instruments

Materials Urea, thiourea, Tris, DTT, double distilled water, TRIzol reagent, chloroform, isopropanol, ethanol, RNase-free water, medium-speed qualitative filter paper (Hangzhou Special Paper Co., Ltd., China), nitrocellulose membranes (NC, 0.22 μm, Whatman PROTRAN nitrocellulose transfer membrane), positively charged nylon 6,6 membranes (0.45 μm, Cat#BNBZF810S, Pall, NY), and plastic film.

Disodium hydrogen phosphate-sodium dihydrogen phosphate buffer preparation: 12.3 ml 1 mol/L Na_2HPO_4 was added with 87.7 ml 1 mol/L NaH_2PO_4, pH adjusted to 6.0 with 5 M NaOH.

Lysis buffer preparation (10 ml): 4.2 g urea, 1.54 g thiourea, 0.05 g Tris, 0.04 g DTT, volume adjusted to 10 ml with double distilled water

Instruments Vacuum pump (type: AP-02B, Tianjin Autoscience Instrument Co., Ltd., China), vacuum filter device (filter cup volume, 300 ml; liquid collection bottle volume, 1000 ml; type, UPI 3S, Tianjin Autoscience Instrument Co., Ltd., China), and multifunctional vacuum packaging machine (DZ-300A).

4.3 Method

4.3.1 Urinary Protein's Preservation Method by Urimem

4.3.1.1 Urinary Protein's Preservation on the NC Membrane

1. Middle stream urine was collected from volunteer. The urine sample was centrifuged in a thermostatic centrifuge for 30 min at 5000 × g and 4 °C, and the supernatant was saved.
2. 15 mL urine and 10 mL buffer solution (1 mol/L Na_2HPO_4, 1 mol/L NaH_2PO_4, pH = 6.0) was mixed together. In the case of proteinuria (protein concentration + ~ ++), 5–10 mL urine was added to 10 mL buffer solution, and mix together.
3. The vacuum suction filter was installed and connected to the vacuum pump.
4. The 0.22 μm NC membrane and the medium-speed qualitative filter papers were cut according to the diameter of filter element (Generally, the diameter of the filter element is 57 mm, and the diameter of the effective filtration area is 42 mm.).
5. Three to four sheets of wetted circular filter paper were placed onto the filter element. And one wetted NC membrane (in pure water) was placed immediately onto the filter papers, avoiding the generation of bubbles.
6. The mixed urine was poured into the funnel.
7. The vacuum pump was opened to allow the urine to pass through the NC membrane dropwise, by adjusting the vacuum pressure, with a velocity of approximately 1~2 droplets/second.
8. After the urinary proteins were adsorbed onto the NC membrane, the protein-bound membrane was placed into 56 °C oven for 4–5 min to completely dry the NC membrane, or left it to dry at room temperature.
9. The dry membrane was sealed in a plastic vacuum bag. Tags were added to the dry membranes that contained the basic information on the sample (medical record number, urine date and time, medication, etc.). And then store it at −80 °C.

4.3.1.2 Elution of the Urinary Proteins from the NC Membrane

1. Nitrocellulose membranes adsorbing urinary proteins were cut into small pieces and transferred into a 2 ml tube. 1.7 mL of acetone and 0.2 mL of 0.5% NH_4HCO_3 added into the tube in turns.
2. The mixture was vortexed intensely at room temperature for 30 s. Then it was heated in 55 °C dry block heater for 60 min and stopped the heating to vortex intensely for 30 s every 20 min.
3. The solution was shaken lightly for 2 h at 4 °C to precipitate the urinary proteins.

4. The solution was centrifuged for 15 min at 18 °C at 12000 r/min. After discarding the supernatant, the precipitation was placed at room temperature for 5–10 min to allow for drying.
5. 300 μL of lysis buffer was added, ultrasonic for 3 minutes after pelleting, and centrifuged at 12000 r/min for 15 min at 18 °C.
6. The supernatant was taken for further analysis. The protein concentration can be measured by Bradford method.

4.3.2 Urinary microRNA Preservation Method by Urimem

4.3.2.1 Urinary Nucleic Acid Preservation on the Nylon Membrane

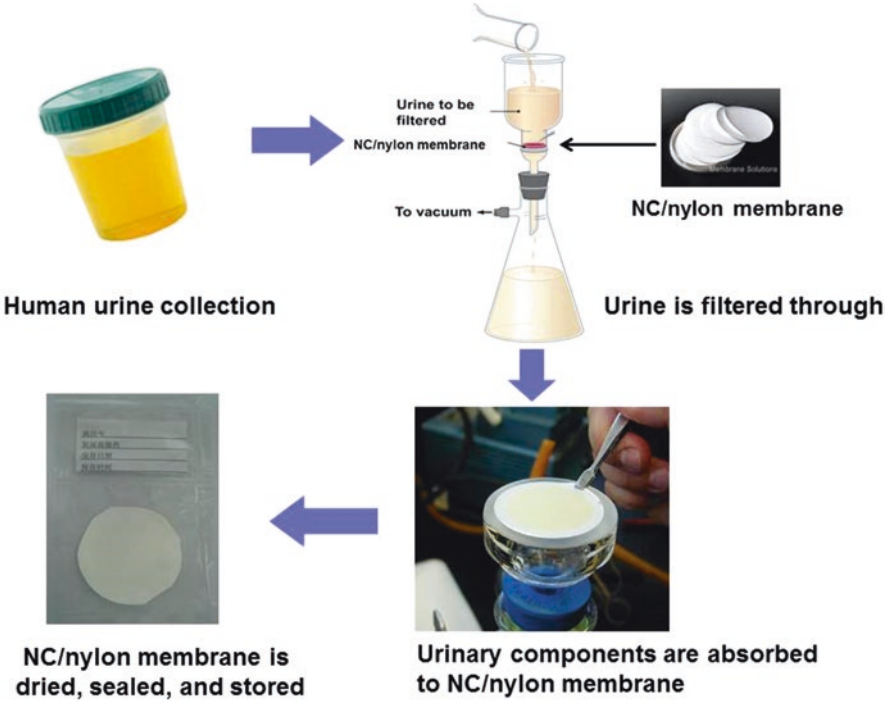
1. Middle stream urine was collected from volunteer. The urine sample was centrifuged in a thermostatic centrifuge for 30 min at 5000 × g and 4 °C, and the supernatant was obtained.
2. The vacuum suction filter was installed and connected to the vacuum pump.
3. The 0.45 μm positively charged nylon 6,6 membranes (Cat#BNBZF810S, Pall, NY) and the medium-speed qualitative filter papers were cut according to the diameter of filter element (Generally, the diameter of the filter element is 57 mm, and the diameter of the effective filtration area is 42 mm.).
4. Three to four sheets of wetted filter paper were placed onto the filter element. And one wetted nylon membrane (in pure water) was placed immediately onto the filter papers, avoiding the generation of bubbles.
5. The urine supernatant was poured into the funnel.
6. The vacuum pump was opened to allow the urine to pass through the nylon membrane dropwise, by adjusting the vacuum pressure, with a velocity of approximately 1–2 droplets/second.
7. After the urinary nucleic acids were adsorbed onto the nylon membrane, the nucleic acid-bound membrane was placed into 56 °C oven for 4–5 min to completely dry the nylon membrane or left it to dry at room temperature.
8. The dry membrane was sealed in a plastic vacuum bag. Tags were added to the dry membranes that contained the basic information on the sample (medical record number, urine date and time, medication, etc.). And then store it at −80 °C.

4.3.2.2 Elution of the Urinary Nucleic Acids from the Nylon Membrane

1. The urinary nucleic acid-bound dry membranes were cut into small pieces and placed into clean tubes. Total RNA, including microRNAs, was extracted by using TRIzol reagent (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions.

2. Briefly, 1 mL TRIzol reagent was added into the tubes with membranes, and then 250 μL of chloroform was added. The sample was shaken vigorously for 15 s and incubated at room temperature for 2–3 min.
3. The sample was centrifuged at 12,000 rpm for 15 min at 4 °C.
4. The fraction from the top aqueous phase was obtained and transferred into new 1.5 mL tubes. And 600 μL of isopropanol was added and then incubated room temperature for 10 min (or − 20 °C overnight).
5. The sample was centrifuged for 15 min at 12,000 rpm at 4 °C.
6. After removing the supernatant aqueous solution, 1 mL of 75% ethanol was added and vortexed to mix.
7. The sample was centrifuged for 10 min at 12,000 rpm at 4 °C.
8. The supernatant ethanol was removed. Dry it for 10 to 15 min at room temperature, and the pellet was obtained. Be careful not to allow the pellet to dry completely; otherwise, it will increase its insolubility.
9. The pellet was dissolved in RNase-free water (10~30 μL) and quantified with a NanoDrop spectrophotometer (Thermo Fisher Scientific). The rest of the sample was stored at −80 °C for further analysis.

4.4 Technical Roadmap



4.5 Discussion

In the last decade, the emergence of "-omic" techniques (proteomic or metabolomic) has greatly accelerated the pace of biomarker research. The intrinsic properties of urine make it an attractive source of biomarkers for clinical omic studies. First, urine is a very suitable sample for collection at home and can even be repeatedly collected from the same individual across the full life cycle. Second, urine is stable and relatively less complex than other biofluids (Decramer et al. 2008; Lee et al. 2008). Third, most importantly, urine has no homeostatic mechanism, a property that makes it accumulate and tolerate huge changes pertaining to the state of our body that we called biomarkers (Gao 2013).

Because urine can enrich changes in all parts of body, it is a highly sensitive matrix which can not only indicate pathological changes in the body but also reflect physiological and pharmacological changes (Wu and Gao 2015). Influence of physiological variations should be excluded when we identified urinary biomarkers. This usually requires us to increase the sample size to achieve statistical performance. Current preservation methods cannot meet the needs of large urine sample storage. As long as we solve the problem associated with rapid and long-term preservation of urine samples, urine can become one of the most promising samples in the precision medicine era.

The current study reported the use of NC/nylon membranes to concentrate and preserve urinary proteins/nucleic acids, respectively. A urine sample could be preserved on a membrane within 5 min or less. It could keep dry in a vacuum bag, thus preventing urinary components' degradation and facilitating interregional sample transfer (Jia et al. 2014; Zhang et al. 2015). The urimem-based preservation method allows for traditional downstream analytical applications. The preserved proteins can be eluted from the membrane for further applications, such as Western blotting or LC-MS/MS analysis (Szewczyk and Summers 1988; Anderson 1985). The nucleic acids preserved on nylon membranes could be extracted for subsequent analysis, such as PCR, real-time PCR, nucleic acid microarray, or other nucleic acid test (Zhang et al. 2015).

At the beginning of the twentieth century, a single concept developed by Dr. Henry Plummer changed the face of medicine. This concept involved a centralized medical record that was stored in a single repository and capable of traveling with the patient. This concept is also applicable to the field of biological sample preservation, and comprehensive storage of biological samples could further revolutionize medical research and practice. Urine is the most promising sample to be "recorded" like a medical history. This simple and inexpensive method makes it possible to begin preserving urine samples from all consenting patients during every stage of disease development or even for those consenting healthy individuals for prospective studies.

However, several factors need to be considered. Samples taken at certain time points should be well-documented in the patient's medical record, and patient consent may be required, both when the sample is collected and when it is analyzed as

part of a particular study. Once urinary component storage becomes an accepted practice by the medical community, technical standards and commercial products will likely be developed. New technologies may emerge, including more durable media with improved adsorption capabilities, streamlined protocols for urinary component collection, drying, sealing, packaging and labeling, and sample storage and management systems for individual sample access and retrieval. Storage at 4 °C or even at ambient temperatures for longer periods may become feasible, while the use of resins might make the preservation of small molecules, including creatinine and certain ions, an economic prospect. Other body fluids, such as cerebrospinal fluid, can also be stored using the same approach.

Comprehensive historical biological information can also be used in both prospective and retrospective studies to improve our understanding of the pathophysiology of certain diseases and potential relationships among diseases or for monitoring the long-term efficacies and side effects of treatments. More ways of extracting and using the information will become evident as increasing numbers of samples become available for research. This information will make medical research easier, faster, and more economic, ultimately benefiting the patients who provided the samples. We believe that this procedure has the potential to change the current landscape of medical research and medical practice.

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Chapter 5
Posttranslation Modifications of Human Urine

Weiwei Qin and Mingshan Wang

Abstract Urine is a promising resource for potential biomarkers. Assessment of the posttranslational modifications (PTMs) is critical when studying protein function/activity, folding, and molecular interactions in relation to disease. Most of the researches on urinary proteome concentrate on the changes of expressional level of proteins. However, many important biological processes are controlled not only by the relative abundance of proteins but also by PTMs. Modification-specific enrichment techniques, coupled with high-resolution mass spectrometry, have greatly enhanced the ability to identify confident PTMs in urine. Enrichment or visualization of proteins with specific posttranslational modifications provides a method for sampling the urinary proteome and reducing sample complexity.

Keywords Posttranslational modifications · Urine · Glycosylation · Phosphorylation · Acetylation

5.1 Introduction

Posttranslational modifications (PTMs) are covalent chemical modifications of proteins and play a vital role in modulating many fundamental biological processes (Mann and Jensen 2003). PTMs determine the spatial structure, charge state, hydrophobicity, and stability of a protein, thereby affecting proteins' activity state, cellular localization, and interactions with other proteins. It makes the structure of the protein more complex, more functional, more precise, and more specific. For example, phosphorylation and dephosphorylation are like a universal switch in the body, almost controlling all life activities in the body (Olsen et al. 2006; Hornbeck et al. 2015). Every human protein has the potential to be posttranslationally modified at least once during its life span. Numerous diseases are driven by the dysregulation of the proteome, due to either changes in expression levels or changes in PTMs. Mass spectrometry (MS)-based proteomics technologies are unrivaled tools for detecting

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and quantifying PTMs. According to the UniProt database, more than 400 PTMs were reported (326 in eukaryotes, 250 in bacteria, and 80 in archaea) (UniProt 2015).

In contrast to the blood, which is controlled by homeostatic mechanisms, urine is an attractive resource for early and sensitive biomarker discovery (Gao 2013; Li et al. 2014). Only a handful PTMs have been studied and applied in urinary biomarker researches (Belczacka et al. 2018; Giorgianni and Beranova-Giorgianni 2016). Compared to more than 400 forms of PTMs detected in proteins, little is known about the PTMs of urine proteins. In this chapter, we focus on three classes of PTMs that had been explored in urine, phosphorylation, glycosylation, and acetylation, and provide examples of relevant studies and results (Table 5.1).

5.2 Glycosylation

Glycosylation is the most frequent PTM, including three distinct types: N-glycans, O-glycans, and glycosaminoglycans (Mechref et al. 2012). It is predicted to affect approximately 50% of the human proteome (Song and Mechref 2015). Glycosylation has a significant impact on multiple biological processes including cell adhesion, signal transduction, and endocytosis. However, glycoproteomic characterization remains difficult due to the complexity and high heterogeneity of glycan.

In 2006, Wang et al. firstly profiled N-linked glycoproteome in normal human urine using concanavalin A enrichment (Wang et al. 2006). A total of 225 urinary proteins were identified based on two-hit criteria with reliability over 97% for each peptide. Among these proteins, 150 were annotated as glycoproteins in Swiss-Prot, and 43 were predicted as glycoproteins by NetNGlyc 1.0. Because the high-abundance protein albumin is not N-glycosylated, the con-A affinity purification could deplete it. Thus, urine N-glycoproteome could provide a more detailed protein profile especially when albuminuria occurs in some kidney diseases. In 2012, Halim et al. firstly reported the attachment sites of glycans on human urinary glycoproteins. In total, 58 N- and 63 O-linked glycopeptides from 53 glycoproteins were characterized with respect to glycan and peptide sequences (Halim et al. 2012). The combination of CID and electron capture dissociation techniques allowed for the exact identification of Ser/Thr attachment site(s) for 40 of 57 putative O-glycosylation sites. In 2018, Belczacka et al. firstly analyzed O- and N-glycosylated intact urinary glycopeptide without applying glycan separation and/or enrichment strategy (Belczacka et al. 2018). A total of 37 intact O-glycopeptides and 23 intact N-glycopeptides were identified in the urinary profiles of 238 normal subjects. In 2018, Kawahara et al. reported the deepest coverage of the N- and O-glycoproteome of human urine (Kawahara et al. 2018). In total, 1310 de-N-glycosylated peptides, 954 intact N-glycopeptides, and 887 desialylated but otherwise intact O-glycopeptides belonging to a total of 788 glycoproteins were identified.

Glycan and glycoprotein expression patterns are considered as an attractive option for the development of novel disease biomarker. In urine, abnormal

Table 5.1 Summary of discovery studies of PTMs in urine

PTM	Disease or condition	MDLC	Enrichment	MS mode	Panel reported	Diseases
Glycosylation	Normal	Y	Y	HCD	194 N-glycosylated proteins	Diabetic nephropathy, bladder cancer, ovarian cancer, prostate cancer, lung cancer, pancreatic cancer, renal cell carcinoma, and cholangiocarcinoma
Glycosylation	Normal	N	N	CID/ ECD	58 N- and 63 O-glycopeptides from 53 glycoproteins	
Glycosylation	Normal and cancers	N	N	HCD/ ETD	37 intact O-glycopeptides and 23 intact N-glycopeptides	
Glycosylation	Pca and BPH	Y	Y	TMT-labled/ HCD	1310 N-glycosylation sites in 729 proteins	
Phosphorylation	Normal	Y	N	CID	45 phosphopeptides/59 sites in 31 proteins	Bladder cancer, Parkinson's disease, clear cell renal cell carcinoma
Phosphorylation	Pregnancy (before/after delivery)	N	Y	CID	130 phosphopeptides/222 sites in 105 proteins	
Phosphorylation	Normal	N	Y	CID	106 phosphosites in 64 proteins	
Phosphorylation	Normal	Y	Y	HCD	4196 phosphosites in 1863 proteins	
Acetylation	Normal	Y	Y	HCD	761 lysine-acetylated peptides/629 sites in 315 proteins	No reports

PCa prostate cancer, *BPH* benign prostatic hyperplasia, *MDLC* multidimensional chromatography, *HCD* high-energy collision dissociation, *CID* collision-induced dissociation, *ECD* electron capture dissociation, *ETD* electron-transfer dissociation, *TMT* tandem mass tag

glycosylation had demonstrated correlation with diabetic nephropathy and cancers. Studies were summarized as follows:

> Urinary transferrin, a glycoprotein with a molecular weight of 76.5 kDa, could reflect the degree of interstitial fibrosis and tubular atrophy in patients with diabetic nephropathy (DN) (Kanauchi et al. 2002). The glycopatterns of (GlcNAc)2–4 showed increased tendency with development of DN (Zhu et al. 2017). Urinary O-glycosylated proteins (clusterin, leucine-rich alpha-2-glycoprotein, and kininogen) are excreted aberrantly on the early stage in ovarian cancer patients (Mu et al. 2013). Glycosylated patterns of A1AT (alpha-1-antitrypsin) may serve as potential biomarkers for early detection (Liang et al. 2015). Davalieva et al. (2015) and Ahmad et al. (2014) showed that N-glycosylation of prostaglandin-H2 D-isomerase (PTGDS) and the O-glycosylation of CD59 are overexpressed in the urine of prostate cancer (PCa) patients. Kawahara et al. complement their observations by reporting that PTGDS and CD59 carry aberrant glycosylation at defined positions in PCa urine (Kawahara et al. 2018). Alterations of sialylated MUC1 glycosylation could be indispensable for the development of cholangiocarcinoma (Matsuda et al. 2015).

5.3 Phosphorylation

Phosphorylation is a common PTM of proteins and occurs most commonly on serine (Ser) and threonine (Thr) residues. According to the manually curated PhosphoSitePlus database (Hornbeck et al. 2015), over 100,000 non-redundant phosphorylation sites were identified on human proteins. One-third of human proteins are phosphorylated or dephosphorylated by an array of kinases and phosphatases (Cohen 2000). Phosphorylation-dephosphorylation events can be very rapid, dynamic, and easily altered during sampling and sample processing. Thus, phosphoproteome is a highly complex and dynamic system.

Li et al. firstly profiled the urinary phosphoproteome using integrated multidimensional liquid chromatography (IMDL) and yin-yang multidimensional liquid chromatography (MDLC) tandem mass spectrometry without prefractionation (Li et al. 2010). The examination yielded 45 unique phosphopeptides containing 59 phosphosites (mostly on serine residues), mapping to 31 proteins. Zheng et al. (2013) reported on quantitative profiling of the urine phosphoproteome in healthy women before and after delivery as a foundation for the discovery of biomarkers for pregnancy-related conditions. In total, 237 phosphopeptides (130 unique phosphopeptides) with 222 phosphorylation sites were confidently identified, representing 105 proteins. Zhao et al. reported the largest dataset of urinary protein phosphorylation, 4196 identified phosphosites from 1863 proteins (Zhao et al. 2018). The in-depth urine phosphoproteome coverage holds promise of new mechanism insights into urine proteome and provides an attractive opportunity that may potentially translate into clinical applications.

Phosphorylation participates in virtually all cellular processes. Changes of cellular protein phosphorylation have been linked to a wide variety of human diseases including cancer, heart disease, obesity and diabetes, and neurodegenerative diseases. However, there's still very little research of phosphorylated proteins in urine.

In bladder cancer, urinary tyrosine phosphoproteins increased approximately five-fold compared to levels in healthy controls (Khadjavi et al. 2011). Elevated ratio of phosphorylated Ser-1292 LRRK2 to total LRRK2 in urine exosomes predicted LRRK2 mutation status and PD risk among LRRK2 mutation carriers (Fraser et al. 2016). The combined evaluation of urinary RKIP and phosphorylated RKIP is a potential diagnostic and prognostic marker of clear cell renal cell carcinoma (Papale et al. 2017).

5.4 Acetylation

Lysine acetylation is an abundant and highly regulated PTM. It was initially discovered in histones approximately 50 years ago (Vidali et al. 1968). Subsequent studies were focused on chromatin remodeling for gene transcription, until the first non-histone protein, p53, was identified to be lysine-acetylated (Gu and Roeder 1997). In 2006, Kim et al. reported the first systematic analysis of lysine-acetylated proteins, indicating that Kac is involved in the regulation of diverse cellular pathways beyond DNA-templated processes (Kim et al. 2006). Subsequent studies detected abundant Kac in non-histone proteins in eukaryotes (such as in human, mouse, and *Drosophila*) (Choudhary et al. 2009; Yu et al. 2009) and prokaryotes (such as *Escherichia coli*, *Salmonella enterica*) (Zhang et al. 2009; Wang et al. 2010). Lysine-acetylated proteins are involved in nearly all cellular processes and are evolutionarily conserved from bacteria to mammals. These proteins are also associated with important diseases such as metabolic diseases, neurodegenerative disorders, and cardiovascular diseases (Menzies et al. 2016; Pons et al. 2009; Voelter-Mahlknecht 2016).

We used anti-acetyllysine antibody-based immunoaffinity enrichment combined with high-resolution mass spectrometry to profile lysine-acetylated proteins in normal human urine. A total of 629 acetylation sites on 315 proteins were identified, including some very low-abundance proteins. This is the first proteome-wide characterization of lysine acetylation proteins in normal human urine. Our dataset provides a useful resource for the further discovery of the lysine-acetylated proteins as biomarker in urine.

5.5 Conclusions and Future Prospects

Urine proteins and their posttranslational modifications have the potential to offer significant insights into physiological and pathological changes in the human body. PTM-centric discovery proteomics can substantially contribute to the understanding of disease mechanisms on the molecular level and changes of PTM patterns correlated with disease, which may help to diagnose or predict the effectiveness of a treatment and guide therapies. However, due to the intrinsically large variability of

urine proteins and their PTMs among different individuals, studying on PTMs is still challenging and far behind that of cell, tissue, or plasma/serum samples. We expect that continued innovations in analytical technologies and informatics tools, together with standardized mass spectrometry assays, drive the biomarker research in urine PTMs.

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Chapter 6
Application of Peptide Level and Posttranslational Modifications to Integrative Analyses in Proteomics

Yongtao Liu and Jianrui Yin

Abstract In a bottom-up strategy, peptide sequences are first identified from MS/MS spectra, and the existence and abundance of the proteins are then inferred from the peptide information. At the same time, posttranslational modifications also play an important role in peptide matching. However, the protein inference step can produce errors and a loss of information. In addition, the genes and proteins are highly homologous in some species, such as human and mouse; if different species of proteins are mixed in one sample, it is difficult to find the difference from protein level alone. In this part, we try to demonstrate the importance of integrative analysis of peptide level and posttranslational modifications in proteomics by two examples.

Keywords Peptide level · Posttranslational modifications · PDX model · Unenriched sample · Open Search

6.1 Introduction

In bottom-up proteomics, the proteins that will be analyzed are enzymatically digested by trypsin into potentially highly complex peptide mixtures; these peptide mixtures are then fractionated using multidimensional liquid chromatography before they are measured with a tandem mass spectrometer (Vizcaino et al. 2013). Subsequently, the existence and abundance of proteins in biological samples are inferred from the peptide information in proteomics studies because the proteins are the executors of organismic functions. Subsequently, the existence and abundance of proteins in samples are inferred from the peptide information in proteomic studies. However, the protein inference step produces errors that inevitably lead to lost

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information (Matzke et al. 2013). On the one hand, even a small false discovery rate (FDR) at the peptide spectrum matches (PSMs) level can translate into a high FDR at the protein level (Nesvizhskii 2010). This effect becomes more pronounced as the number of MS/MS spectra in the dataset increases relative to the number of identifiable proteins in the sample (Nesvizhskii 2010). On the other hand, at the peptide level, the gathered information includes the peptide sequence, hit number, peak area, and peak intensity. When the peptide information is used to synthesize the protein information, only the protein name and the quantitative information remain. This greatly reduces the amount of information, and important information necessary for the comparison of the two samples may be lost. This lost information may provide valuable clues that highlight the differences between the two samples.

In some cases, a proteome sample does not necessarily originate from a single species; it may be protein information containing two or more species, such as parasitism, infection, xenograft, and even contaminated samples. As mentioned above, we want to find out the protein information and composition of different species, usually by retrieving the proteome database of different species to distinguish. However, if there is a high homology between two species, for example, human and mouse, it is difficult to distinguish a protein at the protein level because there may be only a few amino acid residues in amino acid chains. Therefore, it is necessary to identify multispecies mixed proteomics at peptide level.

Information can also be lost if posttranslational modifications (PTMs) are not considered during the database search (Rotilio et al. 2012). This is because only a few preset PTM categories are considered when the peptide information is matched; the software performs protein matching according to these fixed modifications, but the PTMs of the protein is numerous; and currently the selection and retrieval of certain PTMs are another important influence that causes the loss of many peptide information.

Open-pFind, a new generation of open search engine, uses an open search process based on sequence tag indexing to quickly scan protein databases and identify some high-quality spectra. During this process, unexpected modifications, mutations, and semi-specific and non-specific cleavage peptides are all within the engine's search space.

Here, we try to search the database while considering potential PTMs and compare the data at the peptide level to reveal more interesting differences in samples.

6.2 Changes in the Urinary Proteome in a PDX Model at Peptide Level

The urinary proteome from the patient-derived xenograft (PDX) model was examined at the peptide level to study the origins of urinary proteins in tumor-bearing nude mice. Urine was collected from the PDX mice before and after colorectal tumor implantation. A total of 4318 unique peptides were identified, and 78 unambiguous human-origin peptides were discerned in the PDX model urine. This part

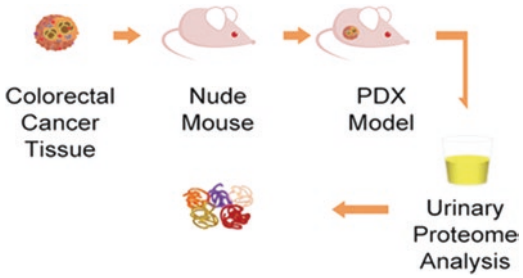

Fig. 6.1 Analysis the species origin of urinary proteins in the PDX model at the peptide level. Colorectal tumor tissues from patients are implanted subcutaneously into nude mice to establish the PDX model. Urine samples are collected from the nude mice before and after tumor transplantation. The samples are analyzed using bottom-up proteome analysis, and the urinary proteins are digested in gel and profiled by liquid chromatography tandem mass spectrometry (LC-MS/MS). The identified peptides are compared at the peptide level

demonstrates that tumor-secreted proteins can be observed in the urine proteome of the PDX model (Fig. 6.1).

Since differential urinary proteins are present in the very early stages of tumor cell implantation (Ni et al. 2018; Wu et al. 2017), it is unlikely that these substantial changes in the urine result from a small number of tumor cells. Therefore, we investigated whether human proteins could be found in the urine of tumor-bearing nude mice. In the patient-derived xenograft model (Tentler et al. 2012; Siolas and Hannon 2013; Hidalgo et al. 2014), human-origin tumors can grow due to the absence of normal T cell immunity. Human-origin peptides that are unambiguously identified in the urine must originate from the human tumor.

Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) was used to compare differences in urinary proteins between the tumor-bearing and control group mice (Fig. 6.2). The urinary proteins were distributed from 10 kDa to 120 kDa in the nude mice, with high-abundance protein(s) at 14 kDa. From this SDS-PAGE result, eight samples were selected from both the tumor-bearing ($n = 4$) and control groups ($n = 4$), and their bands were extracted from the gels. Each lane of the gel was cut into 5 pieces for a total 40 samples. After gel-based digestion, the proteins were digested with trypsin and analyzed by LC-MS/MS.

After processing the data with Proteome Discoverer 2.1 and the Sequest HT algorithm, the MS data (.raw files) were searched against the Homo sapiens and Mus musculus databases to obtain peptide sequences and protein descriptions. In total, 4318 unique peptides were detected from the control and tumor-bearing group samples. After manually comparing the peptides of homologous mouse and human proteins at the same location (https://www.uniprot.org/), 78 human-specific peptides from 42 human-origin proteins were identified. Figure 6.3 shows the main flow of the data analysis.

In the PDX model, 42 unambiguous human-origin proteins (corresponding to 78 human-specific peptides) were found in the urine on day 23 after tumor transplantation, which indicated that they must originate from the human tumor.

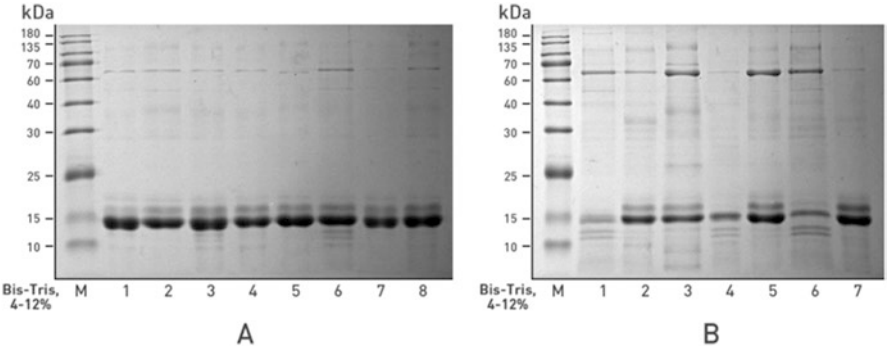

Fig. 6.2 SDS-PAGE analysis of nude mouse urinary proteins. (**a**) Control group (n = 8) and (**b**) tumor-bearing group (n = 7). Protein ladder from 10 kDa to 180 kDa. Method: 80 μg of total protein were loaded into 4–12% Bis-Tris preformed protein gels in SDS running buffer; then, the proteins were separated by electrophoresis at 200 V for 40 min. Each gel line was cut from the bottom up into five pieces based on the concentration

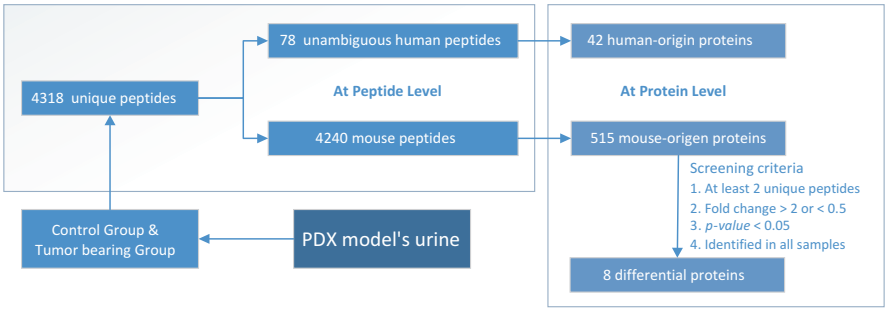

Fig. 6.3 The main flow of the data analysis. The species origin of the proteins was identified first at the peptide level, and then the results were searched for human-origin unambiguous peptides. Subsequently, the next step of the biological analysis was performed at the protein level. Therefore, the peptide level results are converted to protein level results for screening and comparison of differential proteins

Each human-origin protein was searched in the human urinary protein database (https://www.urimarker.com/urine/) (Zhao et al. 2017), which contains information for nearly 6000 normal human urinary proteins. This database is available online and contains the most comprehensive information on human urinary protein biomarkers. By searching this database, the abundance of each tumor-secreted protein in normal human urine was determined, as shown in Table 6.1.

Of these 42 human proteins, 21 were reported in normal human urine, including 16 high-abundance proteins (concentration greater than 1000 pg/mL), 3 moderate-abundance proteins (concentration greater than 100 pg/mL), and 2 low-abundance proteins (concentration less than 100 pg/mL); the remaining 21 proteins did not appear in normal human urine. Half of the 42 human tumor proteins could be

Table 6.1 Human-origin tumor peptides and protein information from the tumor-bearing nude mice

Accession	Protein description	Identification counts	Unique peptide counts	Concentration in human urine (pg/mL)
B4DV14	Highly similar to napsin A	69(4/4)	1	Not found
A1A508	PRSS3 protein (PRSS3)	44(1/4)	1	Not found
D9YZU5	Beta globin (HBB)	33(1/4)	6	73,156.39
P99999	Cytochrome c (CYCS)	26(3/4)	4	235.11
A8K7G6	Highly similar to Homo sapiens regenerating islet-derived 1 alpha	22(1/4)	3	Not found
A0A087WXI5	Cadherin-1 (CDH1)	17(3/4)	4	61,234.54
P48304	Lithostathine-1-beta (REG1B)	14(1/4)	1	Not found
A0A0A6YYJ4	Trefoil factor 3 (TFF3)	13(2/4)	4	13,032.49
P04083	Annexin A1 (ANXA1)	12(1/4)	1	25,421.29
P01037	Cystatin-SN (CST1) (Nandy and Seal 2016)	11(1/4)	5	3490.18
A0A024RAM2	Glutaredoxin (thioltransferase) (GLRX)	9(2/4)	1	Not found
P01036	Cystatin-S (CST4)	7(1/4)	1	4655.98
A0A1K0GXZ1	Globin C1 (GLNC1)	7(1/4)	2	Not found
S6B294	IgG L chain	7(1/4)	1	Not found
H9ZYJ2	Thioredoxin (TXN)	7(2/4)	2	Not found
P36957	Dihydrolipoyllysine (DLST)	6(1/4)	1	4061.93
V9HWA9	Epididymis secretory sperm binding protein Li 62p (HEL-S-62p)	6(1/4)	2	Not found
Q8TAX7	Mucin-7 (MUC7)	6(1/4)	3	Not found
Q6N092	DKFZp686K18196	6(2/4)	3	Not found
Q99988	Growth/differentiation factor 15 (GDF15)	4(2/4)	2	3499.81
A0A1U9X8X6	CDSN	3(1/4)	1	Not found
P04406	Glyceraldehyde-3-phosphate dehydrogenase (GAPDH)	3(1/4)	1	20,395.34
P31151	Protein S100-A7 (S100A7)	3(2/4)	2	1600.63
Q96DA0	Zymogen granule protein 16 homolog B (ZG16B)	3(1/4)	2	14,970.59
Q8N4F0	BPI fold-containing family B member 2 (BPIFB2)	2(1/4)	2	752.72
A9UFC0	Caspase 14 (CASP14)	2(1/4)	2	Not found
Q76LA1	CSTB	2(1/4)	2	Not found
P01040	Cystatin-A (CSTA)	2(1/4)	2	1309.68
Q05DB4	HEBP2	2(1/4)	2	Not found
A7Y9J9	Mucin 5 AC	2(1/4)	2	Not found
Q03403	Trefoil factor 2 (TFF2)	2(1/4)	1	59,910.81

(continued)

Table 6.1 (continued)

Accession	Protein description	Identification counts	Unique peptide counts	Concentration in human urine (pg/mL)
Q13867	Bleomycin hydrolase (BLMH)	1(1/4)	1	262.38
Q8TCX0	Delta 2-isopentenyl pyrophosphate transferase-like protein	1(1/4)	1	Not found
V9HW80	Epididymis luminal protein 220 (HEL-S-70)	1(1/4)	1	Not found
B7Z3K9	Fructose-bisphosphate aldolase	1(1/4)	1	Not found
Q6FH62	HSD17B3	1(1/4)	1	Not found
X6R7Y7	Intraflagellar transport protein 25 homolog (HSPB11)	1(1/4)	1	Not found
Q96P63–2	Serpin B12 (SERPINB12)	1(1/4)	1	Not found
P59665	Neutrophil defensin 1 (DEFA1)	1(1/4)	1	20,375.74
P01833	Polymeric immunoglobulin receptor (PIGR)	1(1/4)	1	129,844.97
A0A158RFU6	RAB7	1(1/4)	1	Not found
P29508	Serpin B3 (SERPINB3)	1(1/4)	1	26,103.15

Note The contents in parentheses after "Identification counts" are expressed as the number of animals/total number of animals per group

detected in normal human urine; therefore, use of these proteins as biomarkers for direct tumor origin at the level of the quantitative proteome when the tumor was relatively large in terms of body weight (Fernandez-Olavarria et al. 2016; Emmink et al. 2013). Table 6.1 provides information for the 42 human proteins, 78 specific peptides, and their concentrations in normal human urine based on a search of the database.

The biological process analysis of the 42 human-origin proteins (Fig. 6.4a) revealed that numerous biological processes were related to digestion and hydrolysis of peptides or proteins (negative regulation of proteolysis, negative regulation of peptidase activity, negative regulation of endopeptidase activity, digestion, and negative regulation of cysteine-type endopeptidase activity) and a response to reactive oxygen species. In the cell composition analysis (Fig. 6.4b), most of the 42 human-origin proteins were derived from the secretion of extracellular exosomes or the extracellular space, region, or matrix rather than from the nucleus or cytoplasm. Molecular functions (Fig. 6.4c) showed that the common functions were related to protein structure and disulfide bonds. These results were consistent with the information about secretion of proteases into surrounding tissues from the tumor during its growth, reproduction, and even invasion. Proteases from a tumor can cause hydrolysis of nearby proteins, which is beneficial for tumor growth and migration. The bioinformatics analysis of the 42 human-origin proteins is shown in Fig. 6.4**.**

The bottom-up proteomics method was used in this study, in which proteins were digested into peptides by trypsin. The MS/MS spectra can only identify peptides. Although humans and mice have very high genetic homology, many tryptic peptides

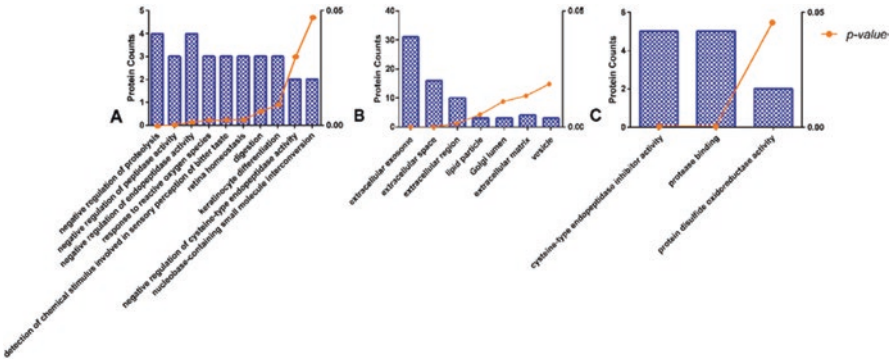

Fig. 6.4 Functional analysis of human-origin tumor proteins in tumor-bearing nude mice. (**a**) Biological process, (**b**) cellular component, and (**c**) molecular function. The ordinate on the left is the protein counts in the relevant pathway, and on the right is the *p-value* of the relevant pathway. A smaller *p-value* indicates a more significant relationship between the pathway and proteins

are not the same for homologous proteins. Some peptides can be identified as human peptides unambiguously. However, biological function information is only available at the protein level. In functional studies, peptides are converted to their corresponding proteins. Therefore, the biological process analysis was carried out at the protein level. Finally, this section explains that the 78 human-origin peptides successfully identified in the urine from the PDX nude mouse model at the peptide level originated from the colorectal tumor.

6.3 Two Leukemia Cell Lines' Proteomic Analysis

To identify the information that is lost using the traditional approaches, this part compared the proteomic data of two leukemia cell lines (Jurkat and K562) at the peptide level with consideration of PTMs. In this study, raw data from two leukemia cell lines, the acute T-cell leukemia Jurkat cell line and the chronic myeloid leukemia K562 cell line, were kindly provided by the Mann Lab (Geiger et al. 2012), and peptide data (Excel files) from the two leukemia cell lines were downloaded from the website http://www.mcponline.org/content/11/3/ M111.014050 (Geiger et al. 2012). The two cell lines underwent LC-MS/MS analysis in triplicate on an LTQ-Orbitrap Velos mass spectrometer and a novel LTQ-Orbitrap family mass spectrometer with a high-field Orbitrap mass analyzer that has double resolution. The label-free quantification of the peptides, with a minimum of two ratio counts, was used to determine the normalized protein intensity (Geiger et al. 2012; Luber et al. 2010), and the data were available for this study in Excel files (Geiger et al. 2012). The raw files from the two cell lines were searched against the decoy IPI-human database version 3.68, which contains forward and reverse sequences. Then the observed modification name in the results was matched with the modification classification on the UniMod website by a manual search. Only the peptides with post-translational modifications were compared between the two cell lines.

To find peptides with differential PTMs between the two cell lines that were not identified using the traditional approach, this study compared the proteomic data from the Jurkat and K562 cell lines at the peptide level while considering the PTMs that may be present. The raw data from two leukemia cell lines (Jurkat and K562) were used in this study because the proteomes and the high-abundance peptides were expected to be similar between the two cell lines. The comparison scheme is illustrated in Fig. 6.5. All of the raw files of the two cell lines were from unenriched samples and were searched by considering 669 modifications using the PEAKS software (experimental procedures). The size of all the raw files was 34.5 GB, and it took 13 days for running PEAKS in a windows server 2008 64-bit OS (CPU: Intel(R) Xeon(R) X5660 @2.80GHz, RAM: 32 GB). Overall, 105,411 and 81,905 non-redundant peptides were identified in the Jurkat and K562 cell lines, respectively, and 44,046 peptides were found in both the Jurkat and K562 cell lines. In the 669 modifications, more than 100 modifications (including about 30 in vivo modifications) with at least four PSMs were found in each cell line. The top eight in vivo modifications in each cell line were hydroxylation, acetylation, dihydroxy, dehydration, carbamylation, methyl ester, sulfone, and phosphorylation.

In MS-based proteomics, it is accepted that side reactions can and do occur on amino acids. In this study, the real in vivo PTMs were isolated from the other PTMs, which include in vitro PTMs and amino acid substitutions, based on the UniMod website. As a result, 7621 and 5763 non-redundant peptides with true in vivo PTMs were found in the Jurkat and K562 cell lines, respectively, from the common 44,046 peptides. The peptides with no more than one peptide spectrum match (PSM) in both cell lines were removed from the result. And the differences from incomplete digestion were eliminated. Fox example, the sequence “AEDGENYDIK” and “AEDGENYDIKR” were considered the same sequence in this study. When the peptides with true in vivo PTMs were compared at the peptide level based on the peptide sequences with PTMs, even without specific PTM enrichment, 5644 peptides had different ways of in vivo modifications in two cell lines. After removing 611 peptides with one PSM in both cell lines, 11.43% (5033/44046) (with at least two spectra in one cell line) were differential peptides, including 4930 peptides that were modified in one cell line but unmodified in another and 764 peptides that were modified in both cell lines with different modification forms or possibly on a different site (Fig. 6.5). For example, the peptide “IKNENTEGSPQEDGVELEGLK” was phosphorylated at the serine residue in the Jurkat cell line, whereas it was not modified in the K562 cell line (Fig. 6.6). Moreover, 1.73% (764/44046) of the peptides were modified in both cell lines but with different types of modifications or on a possibly different site. Only the peptide sequences with PTMs were used to compare the two cell lines, and even more differences would have been found if the peptides had been compared in a quantitative manner. This finding indicates that a comparison of the samples at the peptide level with consideration of PTMs may reveal more differences between the two unenriched datasets. Therefore, database searching with consideration of PTMs is essential.

Numerous raw proteomic data have been searched without consideration of PTMs, particularly data from low-resolution mass spectrometry experiments. Even without

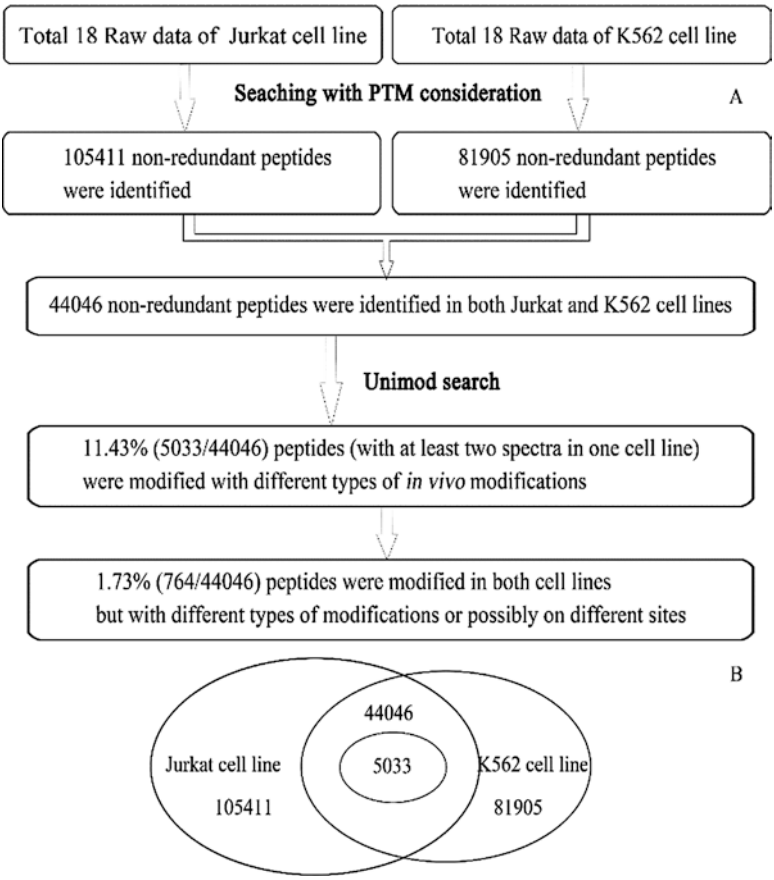

Fig. 6.5 The comparison scheme and the results from the two cell lines at the peptide level when PTMs were considered. All of the raw files of the two cell lines were from an unenriched sample and were searched with the consideration of 669 modifications using the PEAKS software. The real in vivo PTMs were isolated from other PTMs using the UniMod website of these peptides, 11.43% (5033/44046) of them (with at least two spectra in one cell line) existed in different PTM forms, and 1.73% (764/44046) of them were modified in both cell lines but with different types of modifications or possibly on different sites (**a** and **b**)

PTM information in the datasets, some differences will be found only at the peptide level rather than the protein level. Peptide data with no PTMs from the two leukemia cell lines were used in this study. Overall, 86,787 peptides were identified in the two cell lines in the Excel files downloaded from the website. The label-free quantitative information on the peptides was used to perform a t-test and to calculate the FDR. At an FDR level of 0.05, 2136 peptides showed a significant difference between the two cell lines at the peptide level. Although the two cell lines underwent triplicate analysis with different mass spectrometers or resolutions, 2135 of the 2136 differential peptides were identified in all analyses of one cell line but were not identified at all in the other cell line, suggesting that the observed differences were not likely to be random.

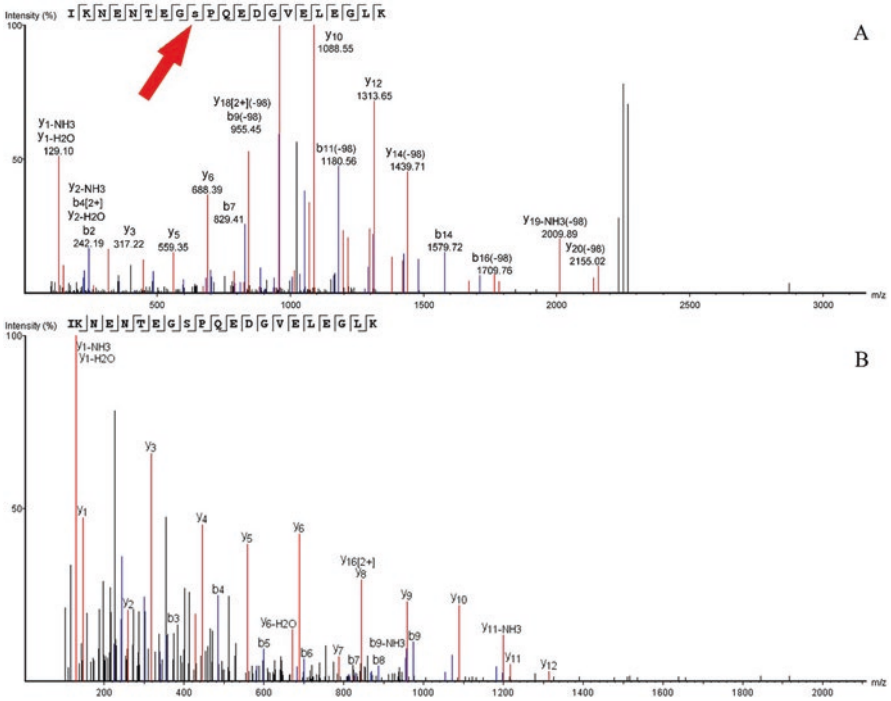

Fig. 6.6 Two different MS/MS spectra of the "IKNENTEGSPQEDGVELEGLK" peptide, which was phosphorylated on the serine residue in the Jurkat cell line (**a**) but was not modified in the K562 cell line (**b**)

The differential peptides were not likely to be caused by the inhibition of high-abundance peptides because all of the differential peptides ranked higher than 15,692 after the peptides were ranked by the decreasing average value of the peptide intensities of the 86,787 peptides, and the highly abundant proteins were similar because the two leukemia cell lines were similar. Moreover, some peptides that were not identified in one cell line were high-abundance peptides in the other cell line.

We observed that there were 16 cell line-specific peptides in 8 protein groups that were identified in both cell lines (Table 6.2), and each protein group had its own peptide in each cell line, indicating that this protein might be structurally and functionally different between the two cell lines. For instance, the peptides "CPIPCIPTIIGTPVK" and "TTVTVPNDGGPIEAVSTIETVPYWTR" were both from the protein group "Rac GTPase-activating protein 1." The former peptide was identified in all triplicate analyses of the Jurkat cell line but was not identified in the K562 cell line; the other way around, the latter peptide was identified in all triplicate analyses of the K562 cell line but was not identified in the Jurkat cell line. According to the UniProt database (http://www.uniprot.org/), the peptide "CPIPCIPTIIGTPVK"

Table 6.2 There were 16 cell line-specific peptides in eight protein groups that were identified in both cell lines

Protein names	Protein IDs	Peptides only in Jurkat	Peptides only in K562
SAPS domain family member 1; serine/threonine-protein phosphatase 6 regulatory subunit 1; SAPS1 protein	IPI00402008; IPI00939427; IPI00873586	VAGAIVQNTEK	VTEPSAPCQAIVSIGDIQATFHGIR
Digestive organ expansion factor homolog	IPI00004290	FGVGDDDDFR	RPEDYEAVFVGNIDDHFR
WD repeat-containing protein 3	IPI00009471	IIIIQGIK	TEVWGIVIISEEK
Male germ cell RacGap; Rac GTPase-activating protein 1	IPI00152946	CPIPCIPTIIGTPVK	TTVTVPNDGGPIEAVSTIETVPYWTR
Hepatocellular carcinoma-associated antigen 56; Ligatin	IPI00013160	GFSVIHTYQDHIWR	NIEAYGIDPYSVAAIIQQR
Munc13–4; protein unc-13 homolog D	IPI00456635; IPI00940441; IPI00790059	IGEITDIHGIR	HSTSAVDISTCFAQISHTAR
Ribosome assembly protein BMS1 homolog; ribosome biogenesis protein BMS1 homolog	IPI00006099	IIAIIDAISTVHSQK	SQIHMPGVGDFAVSDISFIPDPCAIPEQQK
Nuclear-interacting partner of ALK; nuclear-interacting partner of anaplastic lymphoma kinase; zinc finger C3HC-type protein 1; putative uncharacterized protein ZC3HC1; nuclear-interacting partner of anaplastic lymphoma kinase (ALK)	IPI00301421; IPI00945081; IPI00339382; IPI00479595	FQSICHIDIQIPSIRPEDIK	ISIIIHIIEDEIDHR

has been shown to be phosphorylated at threonine residues (Nousiainen et al. 2006; Dephoure et al. 2008). As a result, this protein may have different functions in the two cell lines. These differences also provided us with clues to look for potential PTMs in future studies.

Of the 2136 differential peptides, 52 peptides (with at least two spectra in one cell line) were modified with different types of modifications or possibly on different sites when searched with consideration of PTMs. This finding indicates that even comparing the samples at the peptide level without PTMs may uncover some differences between the samples. However, comparing the samples at the peptide level with consideration of PTMs will reveal more interesting differences, even when the samples are not enriched for PTMs.

6.4 Open Search in Proteomics Analysis

Open-pFind (http://www.pcluster.org/software/pFind3/index.html) is a proteomics data software whose biggest advantage is its ability to perform Open Search on MS/MS-matched PSMs at the peptide level (Chi et al. 2015a; Chi et al. 2015b; Chi et al. 2018) (Fig. 6.7). Compared to most of the current search software, they need to manually select the PTM type of the proteins in the sample data processing, so that the result of the selection is to match the PSMs and peptides in a targeted manner, while the unselected PTM types cannot be matched. This will reduce the information and authenticity of the data obtained. Open Search integrates the UniMod

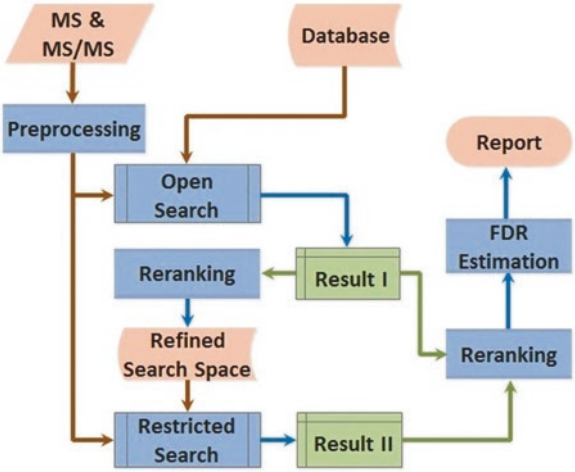

Fig. 6.7 Process for analyzing data using open-pFind. The MS data are first preprocessed by pParse, and then the MS/MS data are searched by the open search module. Next, the MS/MS data are researched by the restricted search module against a refined search space based on the learned information in the reranking step. Finally, the results obtained from both the open and restricted searches are merged, reranked again, and reported

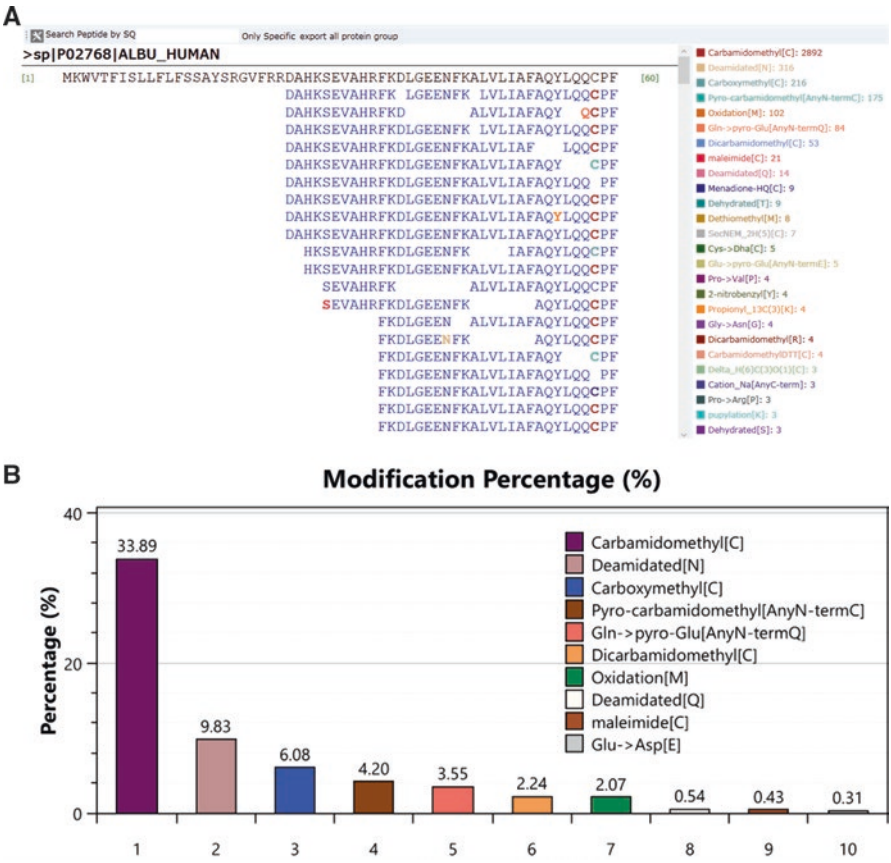

Fig. 6.8 pFind gives the type and number of peptides and posttranslational modifications of albumin in plasma samples (**a**). The proportion of posttranslationally modified peptides in total human plasma samples ranked in the top ten (**b**)

database (http://www.unimod.org) to match different types of known PTMs contained in the database, which not only improves the efficiency of peptide identification but also provides each protein. Posttranslational modification information can help us research on protein results and functional changes at the PTM level (Fig. 6.8).

6.5 Conclusions and Outlook

In PDX model urinary proteome analysis study, the 78 human-origin peptides successfully identified in the urine from the PDX nude mouse model at the peptide level originated from the colorectal tumor. And in two leukemia cell lines' proteomic analysis study, even without enrichment, 5033 differential peptides between

Jurkat and K562 cell lines were found with PTM consideration, and 2136 differential peptides were found at peptide level without PTM consideration. Open-pFind can provide more comprehensive information on PTMs. Therefore, considering potential PTMs and comparing data at the peptide level can reveal more interesting differences between samples, even without specific experimental PTM enrichment. For biologists, it may be more effective and economical to search the database with PTMs than to spend the same amount of time doing experiments. More importantly, some clues found by searching database would hardly be uncovered by current biological experiments. The sensitivity of PTM identification will become greater, and more differences will be found if PTMs are experimentally enriched.

Acknowledgments Part of this chapter is based on published articles: [1] Yongtao Liu, Youzhu Wang, Zhixiang Cao, and Youhe Gao, Changes in the urinary proteome in a patient-derived xenograft (PDX) nude mouse model of colorectal tumor, Scientific report, 2019,9(1): 4975, and [2] Yin, Jianrui, Chen Shao, Lulu Jia, and Youhe Gao, Comparison at the peptide level with posttranslational modification consideration reveals more differences between two unenriched samples, Rapid Communications in Mass Spectrometry, 2014,28 (12):1364-70.

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Chapter 7
Urinary Protein Biomarker Database 2.0: A Literature-Curated Database for Protein Biomarkers in Urine

Chen Shao

Abstract Urine is a valuable source of biomarkers. Current proteomic technologies can identify hundreds of differentially expressed proteins between disease and control samples in a single experiment; however, selection of promise biomarker candidates for further validation study remains difficult. UPBD (Urinary Protein Biomarker Database) was established to collect information of urinary biomarkers or biomarker candidates from published literature in 2011. Both proteomic and non-proteomic studies on all kinds of urine specimens from patients or experimental animals were included in UPBD. To ensure the quality of the database, all research articles were manually curated. This database was updated to version 2.0 in 2017. Standardization of database content was conducted by using terms from several commonly used ontologies and controlled vocabularies. The potential usage of each biomarker (e.g., diagnosis, prognosis) was added as a new field. A new, user-friendly website was developed to provide free browse, search, and download services for nonprofit users. The URL of UPBD 2.0 is http://upbd.bmicc.cn/.

Keywords Urinary biomarker · Database · Literature curation

7.1 The Importance of Urinary Biomarker and Biomarker Database

Biomarkers play a crucial role in all aspects of health care, such as risk assessment, disease diagnosis, prognosis, monitoring, and drug development. The US Food and Drug Administration (FDA) and the National Institutes of Health (NIH) Biomarker Working Group define biomarker as "a defined characteristic that is measured as an indicator of normal biological processes, pathogenic processes, or responses to an exposure or intervention, including therapeutic interventions. Molecular, histologic, radiographic, or physiologic characteristics are types of biomarkers. A biomarker is not an assessment of how an individual feels, functions, or survives" (BEST 2016).

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Urine is among the most valuable biomarker sources. Some protein-based urine tests have been approved by the FDA, such as NMP22 and BTA for bladder cancer and TIMP2 and IGFBP7 for assessing the risk of AKI (Thomas et al. 2016). The FDA has also approved several urinary proteins as nephrotoxicity biomarkers to aid the detection of kidney injury in the process of drug development (FDA's biomarker qualification program, https://www.fda.gov/Drugs/DevelopmentApprovalProcess/DrugDevelopmentToolsQualificationProgram/BiomarkerQualificationProgram/ucm535383.htm).

The state-of-the-art proteomic technologies provide a powerful tool to biomarker discovery. Modern mass spectrometers are capable of confidentially measuring over one thousand proteins in urine within several hours (Leng et al. 2017). As a result, a single proteomic study can identify dozens, if not hundreds, of proteins that have abundance changes in the disease condition. Despite that the measurement of urinary proteome has been greatly accelerated in the past decade, only few urinary proteins have been successfully validated and moved to the stage of clinical application (Thomas et al. 2016). Challenges in this field include, but are not limited to, the complexity and high dynamic range of the urinary proteome and the variability that is caused by many factors not related to disease pathogenesis such as age, sex, hormone status, and method of specimen collection (Harpole et al. 2016).

Despite only a few novel biomarkers have been validated and used in clinical practices in recent years, previous studies still provide rich and valuable information that can be used to aid further biomarker research. In a typical proteomic study, only a small proportion of differentially expressed proteins are selected for further validation, while the remaining ones are ignored from any further investigations and are only reported in a table or a supplemental table in the published literature, which results in insufficient use of the information acquired from the high-throughput experiment. Collecting this information into a database provides the basis of further reuse and mining of it.

In addition, due to the many factors that contribute to the variability of the urinary proteome, a considerable amount of the differentially expressed proteins identified in a proteomic analysis do not reflect the real differences between normal and disease conditions. Enlarging the analyzed sample size might be an ideal solution to eliminate these confounding factors, but it costs too many experimental resources since urinary proteome has been reported to vary even among healthy individuals (Liu et al. n.d.) and is affected by a number of physiological and experimental factors (Oh et al. 2004; Khan and Packer 2006). A biomarker database makes it easy for researchers to conduct cross-study comparisons to find out changes that have been repeatedly observed under the same disease condition on various populations.

Moreover, a biomarker database can facilitate the assessment of disease specificity of biomarkers. Only biomarkers with rigorous disease specificity can be used to distinguish diseases with similar signs and symptoms and to help clinicians to find out the optimized choice of drugs and treatments. By querying a biomarker database, biomarker candidates that are related to multiple diseases can be easily picked

out. Therefore a lot of effort can be saved from validating biomarker candidates that reflect generic changes caused by different categories of diseases.

In summary, a biomarker database provides a convenient tool for cross-study comparison. It can help researchers to assess the confidence and disease specificity of biomarker candidates and allows them to further uncover hidden relationships between diseases and urinary proteins by reanalyzing existing data using novel algorithms and strategies.

7.2 Urinary Protein Biomarker Database 2.0

In 2011, we developed the Urinary Protein Biomarker Database (UPBD) to collect biomarker information from both proteomics and traditional non-proteomic studies from published literature (Shao et al. 2011). Studies with both human subjects and experimental animals were included. Since biomarker information is represented in literature in an unstructured and nonstandard manner, UPBD was designed to be a fully manually curated database in order to guarantee the accuracy of information extraction. UPBD adopts a loose inclusion criterion of "protein biomarkers" as "any protein or its peptide fragment(s) that has been observed to change either qualitatively or quantitatively under a certain physiological or disease condition as compared to normal or baseline." This criterion makes UPBD a comprehensive resource of existing potential biomarkers. When browsing this database, one should keep in mind that "changing under a certain condition" is only necessary but sufficient for a protein to be a biomarker.

In 2017, UPBD was updated to version 2.0 with an improved database schema, new biomarker information from recently published literature, and standardized data content. The new web pages provide user-friendly browsing and searching functions. All biomarker information in this database can be freely downloaded.

7.2.1 Biomarker Descriptions in UPBD 2.0

UPBD 2.0 was designed with the intention to include all necessary elements to describe a biomarker. Variables in this database can be roughly classified into six categories, which are (1) the protein name and ID and (2) the source/matrix. Urine contains several components that are rich of proteins, including the supernatant, small extracellular vesicles (such as exosomes (Street et al. 2017)), and the sediment (containing shed cells and cell components (Oliveira Arcolino et al. 2015; Majewski et al. 2012)); (3) the assays used to discover, validate, and measure the biomarker in laboratory; and (4) the name of disease and (5) the treatment (drug or therapy). This information is only required for biomarkers that are used to predict or assess the effects of treatments and (6) the (potential) clinical or pharmaceutical application of the biomarker (Fig. 7.1).

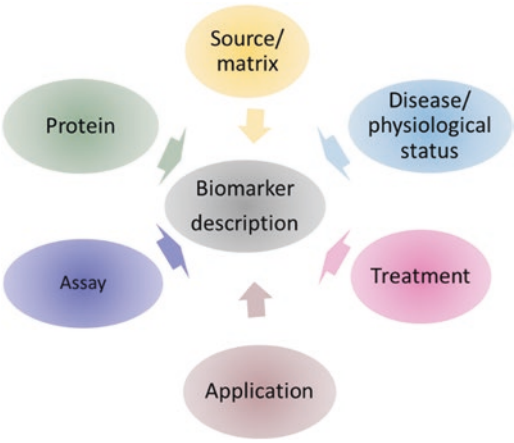

Fig. 7.1 The six major elements of a full biomarker description in UPBD 2.0

Biomarker application is a new field added in UPBD 2.0. Determining the appropriate usage of a biomarker is crucial in biomarker discovery. The FDA-NIH Biomarker Working Group defined seven biomarker categories based on their application (Califf 2018). They are susceptibility/risk biomarker, diagnostic biomarker, prognostic biomarker, predictive biomarker, safety biomarker, pharmacodynamic/response biomarker, and monitoring biomarker. In UPBD 2.0, we expanded these biomarker categories into subclasses to describe more specific biomarker usages. For example, the terms "early diagnosis," "classification," "staging," and "indicator of severity" were added under the category of diagnosis. Note that some studies included in this database only aimed at observing the changes of urinary proteome between disease and normal subjects, the authors did not make any clear statement about biomarker usages. In this situation, the potential biomarker usages of the reported proteins were inferred by our curators.

7.2.2 Standardization of Database Content

Standardization of database content was conducted in UPBD 2.0 by using terms from some widely used ontologies and controlled vocabularies. UniProt was chosen as the reference database for protein names and IDs. Gene symbols of the corresponding proteins were also retrieved from UniProt, so that protein products from homologous genes in different organisms can be linked by their gene symbol. Biomarker information from human and experimental animals were merged into a single dataset. The records of the experimental methods and biomarker applications were standardized by a self-constructed dictionary of controlled vocabularies.

The most difficult work in the standardization of UPBD was to harmonize the highly diverse descriptions of disease and physiological statuses in the literature and to represent them in an organized structure. To solve this problem, we mapped the

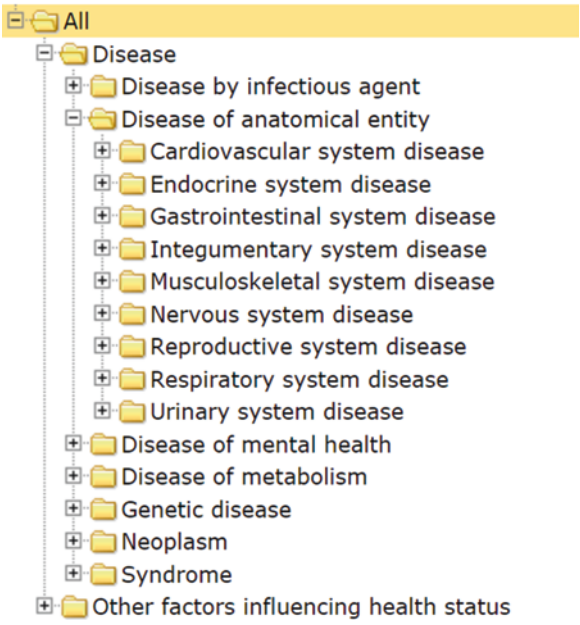

Fig. 7.2 The customized hierarchy representing all terms of disease and another health status in UPBD 2.0

nonstandard descriptions of health statuses that were retrieved from literature to terms from three widely used terminologies, Disease Ontology (DO), National Cancer Institute Thesaurus (NCIT), and International Classification of Diseases, Version 10 – Clinical Modification (ICD10CM), and then integrated them into a customized hierarchy. As shown in Fig. 7.2, all health statuses included in this database are divided into two major categories, disease (using the root term of DO) and other factors influencing health status (using a term from ICD10CM). DO's upper-level disease categories were chosen as the backbone to integrate subcategory disease terms.

7.2.3 The UPBD 2.0 Website

The website of UPBD 2.0 has been moved to http://upbd.bmicc.cn. New web pages were developed to provide improved browsing and searching experiences for users. Till now, UPBD 2.0 includes 1080 biomarker records curated from 691 research articles, covering a diverse category of physiological (such as smoking and pregnant) and disease conditions (Fig. 7.3). The browse page allows browsing the entire database by navigating the tree of health statuses; thus, biomarkers for multiple diseases in the same category can be viewed and compared simultaneously. The experimental evidence for each biomarker is listed on a separate page. For each study, a brief summary of the cohort, experimental method, and result information is shown on this page, making it convenient for users to assess biomarker confidence without checking out the original research articles. Figure 7.4 shows the

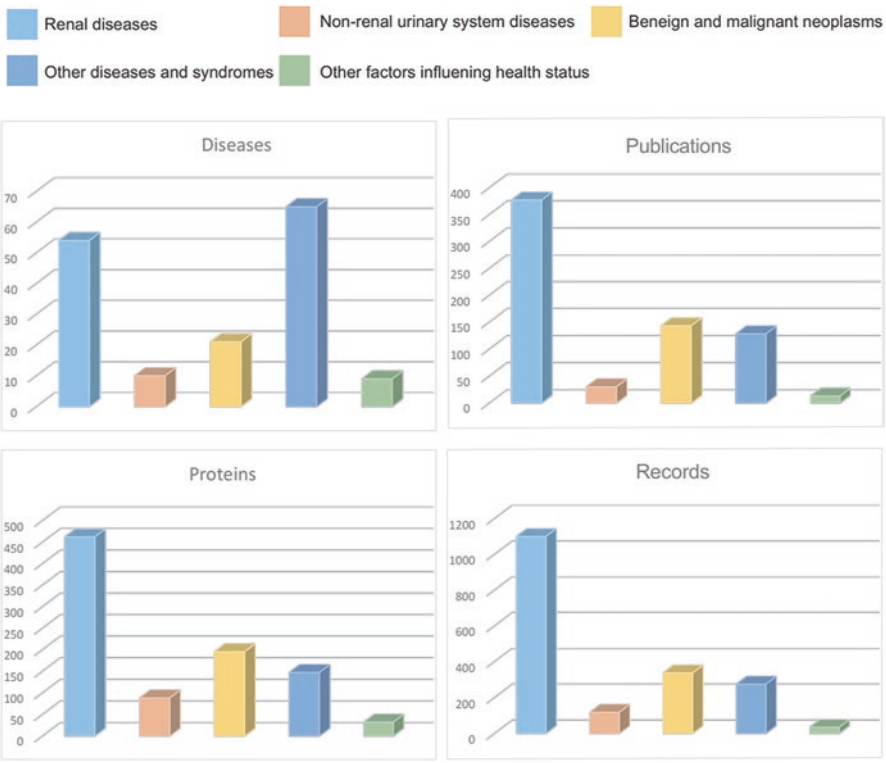

Fig. 7.3 Data statistics

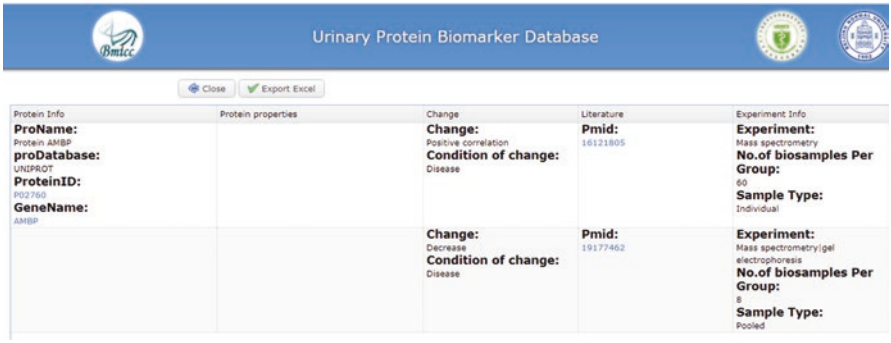

Fig. 7.4 The web page displaying the evidence for protein AMBP as a potential diagnostic biomarker for diabetic nephropathy

evidence of protein AMBP (alpha-1-microglobulin) in urinary supernatant as a potential diagnostic biomarker for diabetic nephropathy. The two publications reported opposite directions of abundance change of this protein under the same disease condition.

The standardization of database content enhanced the development of the new searching page in UPBD 2.0. Users can query the database by any feature of a biomarker (e.g., disease, protein name, biomarker usage, specimen type) or by any combination of these features. Multiple restrictions such as assays used to detect the biomarker, the minimum number of biological replicates per group, and the type of sample (individual or pooled) can also be added to the query. The result of the query is displayed in a table that can be freely downloaded. The searching page provides a powerful tool for researchers who want to find literature evidence for potential biomarkers discovered in their own research or to check the disease specificities of them.

7.3 Perspectives

Standardization is a crucial process in the establishment of a biomarker database. In UPBD 2.0, we made use of terms from some commonly used ontologies and terminologies to standardize the storage and representation of biomarker information. All biomarker information is now stored in a SQL database model to support the browsing and searching services of the website. However, UPBD needs to be more interoperable with other public databases and data analysis tools in order to make better use of its data. For example, UPBD 2.0 used the seven categories of biomarker application defined by the FDA-NIH Biomarker Working Group; however, other documents defined slightly different biomarker types (Anderson and Kodukula 2014). Harmonization of every concept with other widely used databases and standardization of every data entity is the future work of UPBD. This work can benefit from using related biomedical ontologies and building a new ontological model for information representation (Ceusters and Smith 2015; Buckler et al. 2013; Bandrowski et al. 2016).

Since established in 2011, biomarker records in UPBD have been updated several times to include the latest research findings in this database. This update process cost a lot of effort since our curators had to read through the full text of each article to ensure biomarker information has been correctly extracted. Text mining has become an emerging field in biomedical research. Researchers have established pipelines for automated or semiautomated biomarker information retrieving (Jordan et al. 2014; Bravo et al. 2014). Recently, deep learning algorithms demonstrated significant improvement in biomedical name entity recognition (Zhu et al. 2018; Habibi et al. 2017), providing the fundamental tools for predicting the relationships

between proteins and diseases. Existing text mining tools are mainly for mining disease-molecule associations. Semantic recognition in the result or conclusion sections stating “a certain protein is a potential biomarker for a disease” is the most difficult task in text mining, which may need the assistance of manual curation.

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Part II
Urinary Biomarkers of Diseases

Chapter 8
Urine Glucose Levels Are Disordered Before Blood Glucose Level Increase Was Observed in Zucker Diabetic Fatty Rats

Wei Yin and Weiwei Qin

Abstract Many patients with diabetes are not diagnosed at all or are diagnosed too late to be effectively treated, resulting in nonspecific symptoms and a long period of incubation of the disease. Pre-diabetes is an early warning signal of diabetes, and the change of urine glucose in this period has been ignored even though urine has long been related with diabetes. In this study, Zucker diabetic fatty (ZDF) rats were used to test if there were changes in urine glucose before blood glucose increases. Six 8-week-old male ZDF rats (fa/fa) and Zucker lean (ZL) rats (fa/+) were fed with Purina 5008 high-fat diet and tested for fasting blood glucose and urine glucose. After 12 weeks of feeding, the urine glucose values of the ZL rats were normal (0–10 mmol/L), but the values of the ZDF model rats increased 10 weeks before their blood glucose levels elevated. The urine glucose values of the ZDF model rats showed a state of disorder that was frequently elevated (>10 mmol/L) and occasionally normal (0–10 mmol/L). This finding may provide an easy early screening for diabetes by long-term monitoring of urine glucose levels: pre-diabetes may be revealed by frequently disordered urine glucose levels over a period.

Keywords Type 2 diabetes mellitus · Urine glucose · ZDF rats · Impaired glucose regulation · Prediabetes

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8.1 Introduction

According to a survey by the World Health Organization, the number of global diabetic patients increased from 108 million in 1980 to 422 million in 2014, which contained 4.7% of the world's adults in 1980 and 8.5% in 2014 (W.H.O 2016). Currently, China has the largest number of diabetic patients, approximately 114 million, with 11.6% of adult men suffering from diabetes, of which approximately 90% is type 2 diabetes mellitus; by 2040, the number may increase to 150 million (Xu et al. 2013). Without ideal treatment, diabetes may cause many complications such as cardiovascular and cerebrovascular disease; kidney, retinal, and nervous chronic diseases, and various infections; even severe ketoacidosis and disability or death which is the worst may occur (Danaei et al. 2014; Seuring et al. 2015).

The early symptoms of diabetes are not obvious. The disease has a long incubation period; therefore, many patients did not even realize they had developed the disease. A large part of them had contracted complications in the brain, heart, eye, kidney, and other important organs, which were the main reasons for the high disability and mortality rate of diabetes, which burdens both the patients and the healthcare system. The early diagnosis of diabetes is the prerequisite for early intervention, which is very necessary to reduce the burden (Tabák et al. 2012).

Pre-diabetes mellitus, also known as impaired glucose regulation (Bansal 2015), is an intermediate state of diabetes in healthy people, which is considered a necessary stage and an early warning signal of diabetes. Impaired glucose regulation contains impaired fasting glucose and impaired glucose tolerance (Alberti and Zimmet 1998; W.H.O. 1985). Impaired glucose tolerance refers to the special metabolic state with slightly higher postprandial blood glucose levels between healthy and diabetic patients, while fasting blood glucose levels are normal. Currently, it is believed that impaired glucose regulation is an early manifestation of the pathogenetic process of diabetes, especially in type 2 diabetes mellitus. The pre-diabetes patient number in China may reach 493.4 million, which has become a serious public health problem (Yang et al. 2012).

The 2016 American Diabetes Association guidelines for the diagnosis and treatment criteria of pre-diabetes are as follows: impaired fasting glucose of fasting blood glucose from 5.6 to 6.9 $mmol \cdot L^{-1}$ and impaired glucose tolerance of 2-h postprandial blood glucose from 7.8 to 11.1 $mmol \cdot L^{-1}$ (A.D.A. 2016). Pre-diabetes is the important transitional stage from normal glucose tolerance to diabetes. However, at the same time, pancreatic β cells of impaired glucose tolerance patients still maintain some compensatory capacity, which significantly delays or even prevents the majority of occurrence of diabetes through reasonable intervention. Therefore, rapid, convenient, and effective diagnosis of pre-diabetes is critical to preventing and controlling diabetes (Bansal 2015).

Despite increasing awareness of the risk factors for type 2 diabetes and cases of successful preventive methods, the incidence and prevalence of the disease continues to rise globally. Early detection and screening as well as safe and effective treatments can prevent or delay complications to reduce morbidity and mortality. Knowledge of specific diabetes phenotypes and genotypes will lead to more specific and individualized management of type 2 diabetes patients. Traditional tests for diabetes, such as fasting

blood glucose, 2-h postprandial blood glucose, glycosylated hemoglobin, and insulin resistance, are widely used for routine diagnosis and risk grading of type 2 diabetes. However, when these traditional indicators are found to be abnormal, diabetes may have occurred for many years and complications may occur. Therefore, finding the metabolic patterns of the occurrence and development of type 2 diabetes and biomarkers reflecting changes in early metabolic indicators not only contribute to the understanding of the etiology of the disease but also can provide a theoretical basis for early diagnosis, self-management, and prevention strategies for type 2 diabetes.

Individuals at risk for type 2 diabetes must be screened to minimize the occurrence and progression of microvascular and macrovascular complications. It is not easy to advocate universal screening because the results of large randomized controlled trials show that centralized management of screening patients does not improve cardiovascular risk or other outcomes (Webb et al. 2011; Simmons et al. 2012). Screening for risk assessment verification is currently recommended, ideally suited for different countries and subpopulations (Khunti et al. 2015), but for the public, fasting blood glucose and impaired glucose tolerance testing is too complex to monitor daily at home, and the process of measuring blood glucose is not comfortable for everyone, especially before a definite diagnosis. In contrast, the measurement of urine glucose, which is simple and noninvasive, is more convenient and highly accepted, and it can be repeated more frequently at any time.

The elevated urine glucose level is an important feature of diabetes, leading to the discovery of diabetes in the beginning (Poretsky 2010). But all this time people share a concept that the elevated urine glucose level is associated with the elevated blood glucose level. The change of urine glucose before blood glucose level increases has been ignored. So we choose an animal model to study the change of urine glucose in earlier period of type 2 diabetes mellitus.

In this study, ZDF rats were used to study the change of urine before type 2 diabetes mellitus diagnosis to find a convenient, noninvasive early indicator of impaired glucose regulation.

8.2 Materials and Methods

8.2.1 Animal Experiments

This study consisted of six male ZDF rats (fa/fa) and six male ZL rats (fa/+). Animals were received from Beijing Vital River Laboratory Animal Technology Co., Ltd. (Beijing, China) at 8 weeks of age and were individually caged to allow for individual measurements of food consumption. All animals were fed with Purina 5008 rat chow (protein = 23%, carbohydrate = 58.5%, fat = 6.5%, fiber = 4%, and ash = 8% by weight). All rats were maintained under stringent environmental conditions that included strict adherence to 12-h light/dark cycles. All animal manipulations and care procedures were carried out between 1.5 and 3.5 h after lights on. Animals were fed ad libitum from 8 to 24 weeks of age and then sacrificed by aortic exsanguination. All protocols adhered to the "Guide for the Care and Use of Laboratory Animals" and were approved by our institution's Institutional Animal Care and Use Committee.

8.2.2 Experimental Design

Once a week, rats were placed individually in metabolic cages fasting for 12 h; the urine was collected in constant animal room temperature at 25 °C, stored at 4 °C every 3 h, and finally mixed together. Then, urine volume and body weights were recorded, and blood was sampled from the retrobulbar venous plexus for analysis of fasting blood glucose measured with glucometer (ACCU-CHEK®, Roche Diabetes Care, Inc., Indianapolis, Indiana). Urine glucose concentrations were analyzed with a urine glucose assay kit (oxidase method, Beijing Applygen Technologies Inc., Beijing, China).

8.3 Results

8.3.1 Metabolic Parameters

During the treatment period of 16 weeks (age of the rats: 8–24 weeks), there were no spontaneous deaths in all animals. ZL rats showed good growth, flexible activities, and healthy coat color. ZDF rats had increased feed and water intake, increased urine output, and excrement and dull coat color. ZDF rats showed a significant increase in body weight ($P < 0.05$), which increased rapidly from 8 to 12 weeks and slowly after 12 weeks. ZL rats showed a continuous and stable increase. The body weight changes of ZDF rats and ZL rats are shown in Fig. 8.1.

8.3.2 Glucose Changes

The fasting blood glucose level of ZDF rats increased obviously from 16 weeks and was significantly higher than ZL rats ($P < 0.05$), as shown in Fig. 8.2. The urine glucose concentration of ZL rats was always between 0 and 10 $mmol \cdot L^{-1}$. The concentration in ZDF rats was occasionally lower than 10 $mmol \cdot L^{-1}$, but most of the time, it was higher than 10 $mmol \cdot L^{-1}$. The concentration is always higher than 10 $mmol \cdot L^{-1}$ when the fasting blood glucose level turned to rapid elevation phase, as shown in Fig. 8.2.

8.4 Discussion

The kidney is the main excretory organ; most of the glucose in glomerular filtrate will be reabsorbed back into the blood by renal tubules in healthy individuals. Only a very small amount of glucose remains in urine, which cannot be detected in a

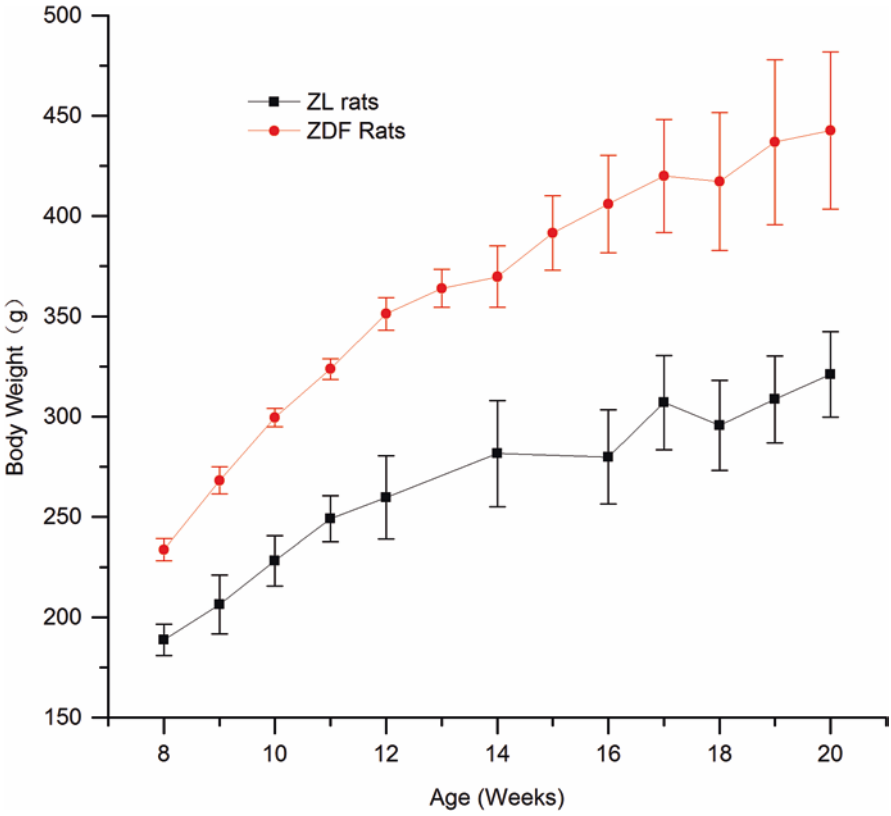

Fig. 8.1 The body weight changes of ZDF rats and ZL rats. Body weight of ZDF rats (red circles) and ZL rats (black squares) (n = 6, mean ± SEM, fed conditions). Symbols represent means, and error bars represent one standard deviation. ZDF rats were significantly different from ZL rats ($P < 0.05$)

urine test. However, the glucose reabsorption by proximal tubules is limited. When blood glucose concentration is more than 8.96–10.08 mmol/L, epithelial cells of proximal tubule reach the limit of glucose absorption, and thus glucose cannot be fully reabsorbed and excreted into urine, which is called glycosuria. The lowest blood glucose concentration at which glucose begins to appear in urine is known as renal threshold for glucose (RT_G). When the blood glucose concentration exceeds the RT_G, urine glucose begins to appear (Polidori et al. 2013a, b).

Although the blood glucose level of ZDF rats is higher than ZL rats, it is still in the normal range and no higher than the RT_G of ZDF rats (Liang et al. 2012). Before crossing the key point of elevated blood glucose, the increasing urine blood level may be due to impaired glucose regulation, which caused short-term and uncaptured changes when blood glucose level exceeds the RT_G. As urine glucose is monitored over a period, unlike in blood that is monitored at a point of time, urine glucose is easily captured, and the disorder of urine glucose level can be observed. Previous

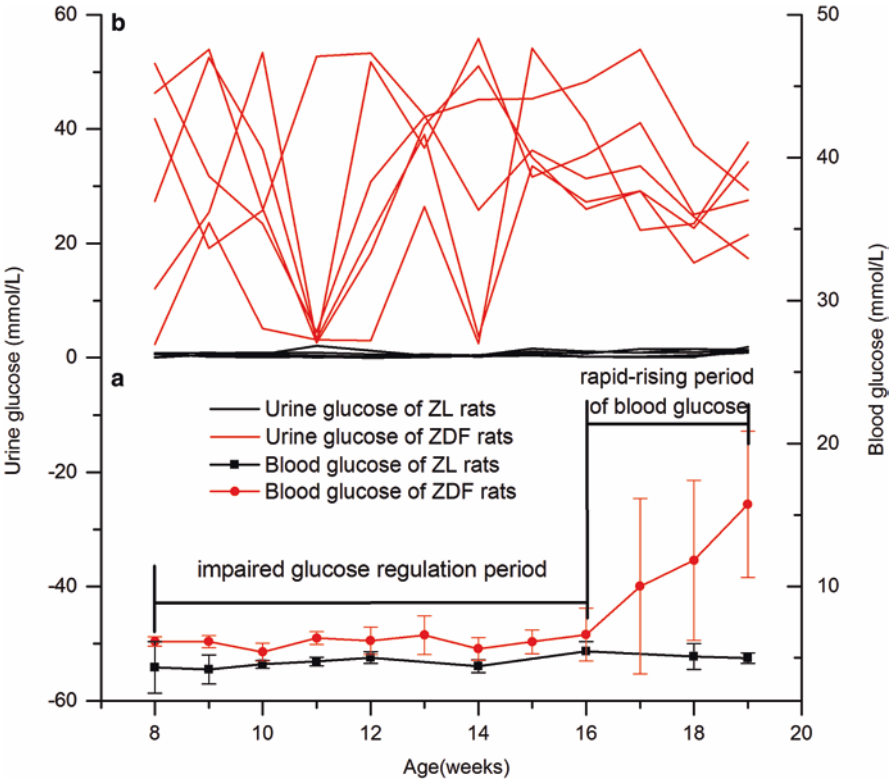

Fig. 8.2 Fasting blood glucose and urine glucose changes of ZDF rats and ZL rats. (**a**) Fasting blood glucose of ZDF rats (red circles) and ZL rats (black squares) (n = 6, mean ± SEM, fed conditions). Symbols represent means, and error bars represent one standard deviation. ZDF rats were significantly different from ZL rats after week 16 ($P < 0.05$). (**b**) Urine glucose concentration of ZDF rats (red lines) and ZL rats (black lines) (n = 6, fed conditions). Urine glucose level of ZDF rats were disordered and significantly different from ZL rats, which were always low

studies have produced similar findings (Hempe et al., 2012) regarding urine glucose disorder before blood glucose goes beyond the normal range and regarding how it may be a valuable early warning sign.

Based on the results of this study, during early impaired glucose regulation, urine glucose disorder may be caused by occasional blood glucose increase over the RT_G at some points. When glucose is filtered into the urine, glycosuria begins to appear. Because of the ability of blood to maintain steady state is strong, blood glucose is normal most of the time, especially after 12 h of fasting. During impaired glucose regulation, blood glucose increases, which reflects a status of a certain time point and may be difficult to capture. However, since urine can accumulate for a period, the glucose increase can be captured easily in urine. Therefore, in the pre-diabetes period, urine can promptly reflect the appearance of impaired glucose regulation by the disorder of urine glucose levels.

It is likely that the more serious impaired glucose regulation becomes, the greater the frequency of abnormal urine glucose levels tend to be. When the fasting blood glucose concentration increases, urine glucose levels stay high. This phenomenon provides a new thought for the early warning of type 2 diabetes mellitus, suggesting that human diabetes screening can be carried out by frequent urine glucose monitoring. If the above findings remain true in humans, the frequency of high urine glucose level may be an indicator to impaired glucose regulation, which can be used for simple, noninvasive, and effective home self-monitoring. Intelligent closestools, which can provide easy frequent urine glucose testing at home, may help to identify pre-diabetes people.

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Chapter 9
Cancer Biomarker Discovery in Urine of Walker 256 Tumor-Bearing Models

Jianqiang Wu, Jing Wei, Linpei Zhang, Ting Wang, and Yameng Zhang

Abstract Urine accumulates systemic changes in the body without homeostatic control; thus, it has the potential for early detection of cancer. In this chapter, five tumor-bearing models were established by injection of Walker 256 tumor cells into the subcutaneous, lung, brain, liver, or bone cavity of rats. Urine samples were collected at multiple time points after tumor cell inoculation. Dynamic urine proteomes were analyzed using label-free relative quantification. We found the urinary protein patterns changed significantly with cancer development, and some urinary proteins even changed at an early onset of tumor growth. Moreover, urine proteomics could differentiate the same cancer cells grown at different organs. We think that urine is a noninvasive and promising source in cancer biomarker discovery especially in the early phase.

Keywords Urine · Proteomics · Cancer biomarkers · Early detection · Monitoring

9.1 Introduction

Cancer biomarkers are measurable changes associated with the pathophysiological processes of cancers. Cancer biomarkers can be biological molecules which are either produced by cancer cells or by the body as a response to the presence of the tumor. They can provide diagnostic, prognostic, or predictive information. Urine is a promising bio-fluid for biomarker research. Without homeostatic control, urine can accumulate systemic changes in the body. Thus, small and early pathological changes can be reflected in urine (Gao 2013; Wu and Gao 2015). In addition, urine can be collected in a noninvasive manner, and it is easy to repeatedly collect large amounts of urine from patients. Recently, urinary proteomics has been applied to biomarker discovery in various cancer diseases (Husi et al. 2011; Zoidakis et al.

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2012; Zhang et al. 2018a; Sandow et al. 2018). However, it is unclear whether urine proteins could enable the sensitive detection of cancer in the early stages of tumor growth.

To limit the effects of confounding factors, we can use animal models to establish the direct relationship between urinary protein changes and cancer progression (Zhao 2015; Yuan et al. 2015). In animal models, effects of genetic and environmental factors on the urine proteome are limited to the minimum. Moreover, the exact starting point of tumor growth is available, which is very helpful in the identification of early cancer biomarkers. In addition, the interference of drug treatment on urine proteome can be avoid in animal models.

In this chapter, five tumor-bearing rat models were, respectively, established by injection of Walker 256 (W256) tumor cells into the subcutaneous, lung, brain, liver, and bone cavity. Urine samples were collected at multiple time points after the rats were placed in metabolic cages individually. Urine proteomes of tumor-bearing models were analyzed by LC-MS/MS and label-free quantification.

9.2 Changes of Urine Proteome in W256 Subcutaneous Tumor Model

The subcutaneous tumor-bearing rat model was established using a previously reported method (Shen et al. 2011). Briefly, after two cell passages of W256 tumor cells in ascitic fluid, 2×10^6 viable W256 cells were subcutaneously inoculated into the right flank of male Wistar rats (150 ± 20 g). After tumor-bearing rats were placed in metabolic cages individually, urine samples were collected on days 0, 4, 6, 9, 11, and 14. Urine proteins were precipitated with ethanol and resuspended in lysis buffer. Urine proteins were digested with trypsin using the FASP method. Peptide mixtures were desalted and analyzed with LC-MS/MS using an Orbitrap Fusion Lumos Tribrid mass spectrometer. Urine protein patterns of a tumor-bearing rat at multiple tie points were displayed. As shown in Fig. 9.1, the urinary protein patterns of subcutaneous tumor-bearing rats changed significantly with the tumor progression.

At the biomarker discovery phase, a total of 20 urine samples were collected at five time points (days 0, 4, 6, 9, and 14) from four tumor-bearing rats and used for label-free proteome quantification. As a result, 533 urine proteins were identified with ≥ 2 unique peptides. After clustering analysis of these urinary proteins at multiple time points, it was observed that samples at each tumor stage were almost clustered together (Fig. 9.2a). Then the differential expression of urinary proteins with tumor progression was analyzed, and differential urine proteins were identified. The screening criteria included fold change ≥ 1.5 and $P < 0.05$ compared with day 0; protein spectral count of each sample in the high-abundance group greater than that in the low-abundance group; and average spectral count of high-abundance group more than 4. The overlap of differential urine proteins identified at different time points is shown in Fig. 9.2b. Interestingly, differential proteins identified at the early phase (day 4) could also be identified with differential abundances at later tumor stages.

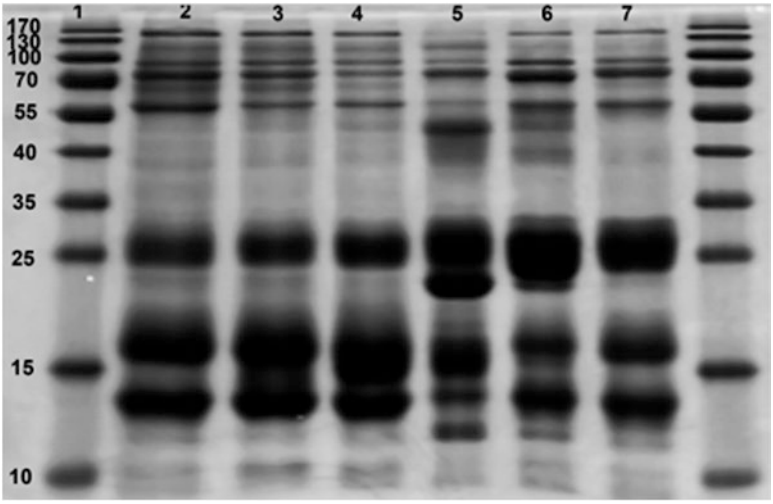

Fig. 9.1 Dynamic changes of urinary protein patterns of a subcutaneous tumor-bearing rat. Lane 1: Marker (10–170 kDa). Lanes 2–7: Days 0/4/6/9/11/14 after W256 cell inoculation. This figure is cited from (Wu et al. 2017)

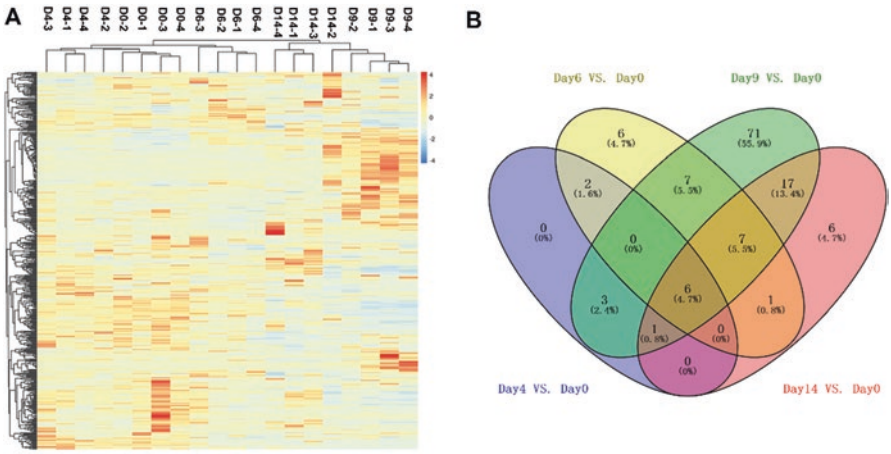

Fig. 9.2 Proteomic analysis of urine samples of subcutaneous tumor-bearing rats at five time points. (**a**) Clustering analysis of urine proteins identified by LC-MS/ MS. (**b**) Overlap evaluation of differential urine proteins at different time points. This figure is cited from (Wu et al. 2017)

Then, 30 dynamically changed differential proteins were used for biomarker validation (Table 9.1). Urine samples from another four model rats were performed for multiple reaction monitoring (MRM) assay. As a result, a total of 20 differential proteins dynamically changed at multiple time points by MRM analysis (Fig. 9.3). Expression trends of these urine proteins were consistent with the label-free

Table 9.1 Differential urinary proteins selected for MRM validation

UniProt	Protein description	Trends	*P* values	Average fold change			
				Day 4	Day 6	Day 9	Day 14
P07151	Beta-2-microglobulin (B2MG)[a]	↑	0.006	3.22	4.37	8.33	4.33
P02764	Alpha-1-acid glycoprotein (A1AG)	↑	0.007	1.81	2.49	6.05	1.77
P06866	Haptoglobin (HPT)	↑	0.007	2.88	5.06	3.31	3.56
P08649	Complement C4 (CO4)[a]	↑	0.028	3.40	4.66	6.14	3.57
O70513	Galectin-3-binding protein (LG3BP)[a]	↑	<0.001	6.77	4.55	2.32	1.55[b]
P29534	Vascular cell adhesion protein 1 (VCAM1)[a]	↑	<0.001	1.45[b]	1.73	2.58	2.00
Q8JZQ0	Macrophage colony-stimulating factor 1 (CSF1)[a]	↑	0.063	2.08	2.08	2.31	1.92[b]
P30152	Neutrophil gelatinase-associated lipocalin (NGAL)[a]	↑	0.005	1.06[b]	2.12	19.06	3.18[b]
Q4V885	Collectin-12 (COL12)	↑	0.087	2.50	3.25	3.13[b]	1.75[b]
P47967	Galectin-5 (LEG5)	↑	0.023	1.37[b]	1.59	3.52	3.07
P01048	Cluster of T-kininogen 1 (KNT1)	↑	0.033	1.36[b]	1.97	3.61	3.03[b]
P97840	Galectin-9 (LEG9) [a]	↑	0.022	5.5[b]	6[b]	13.50	14.00
P20761	Ig gamma-2B chain C region (IGG2B)	↑	0.053	3.00[b]	1.00[b]	46.00	62.00
P10758	Lithostathine (LITH)	↑	0.030	∞	∞	∞	∞[b]
P02651	Apolipoprotein A-IV (APOA4) [a]	↓	0.005	0.36	0.00	0.00	0.14
P80067	Dipeptidyl peptidase 1 (CATC) [a]	↓	<0.001	0.63	0.63	0.53	0.41
P04276	Vitamin D-binding protein (VTDB) [a]	↓	0.031	0.59	0.41	0.26	0.40[b]
Q99J86	Attractin (ATRN)	↓	0.002	0.87[b]	0.58	0.13	0.53
P02454	Collagen alpha-1(I) chain (CO1A1) [a]	↓	<0.001	0.75[b]	1.28[b]	0.16	0.09
Q0PMD2	Anthrax toxin receptor 1 (ANTR1) [a]	↓	0.004	0.79[b]	0.89[b]	0.14	0.39
P48199	C-reactive protein (CRP) [a]	↓	<0.001	1.10[b]	0.43	0.17	0.45
Q00657	Chondroitin sulfate proteoglycan 4 (CSPG4)	↓	0.004	0.90[b]	0.71	0.24	0.36
Q9QZA2	Programmed cell death 6-interacting protein (PDC6I) [a]	↓	0.005	0.37	0.33	0.04[b]	0.07[b]
Q63772	Growth arrest-specific protein 6 (GAS6) [a]	↓	0.010	0.83[b]	0.58	0.13	0.33
P07171	Calbindin (CALB1)	↓	0.045	0.51	0.57[b]	0.31	0.00
P08289	Alkaline phosphatase, tissue-nonspecific isozyme (PPBT) [a]	↓	0.004	0.85[b]	0.45[b]	0.20[b]	0.30
D3ZTE0	Coagulation factor XII (FA12)	↓	0.002	0.42	0.53	0.05	0.00
Q9R0D6	Transcobalamin-2 (TCO2)	↓	0.044	0.75[b]	0.40	0.30	0.30
P07897	Aggrecan core protein (PGCA)	↓	0.003	0.83[b]	0.97[b]	0.13	0.20
Q9EQV6	Tripeptidyl-peptidase 1 (TPP1)	↓	0.049	0.94[b]	0.44	0.75[b]	0.38

Average fold change is the average value from all four rats compared with day 0

[a]These proteins are cancer biomarkers annotated in the IPA database or urine biomarker candidates of cancer patients

[b]No statistical significance compared with day 0 ($P > 0.05$). This table is cited from (Wu et al. 2017)

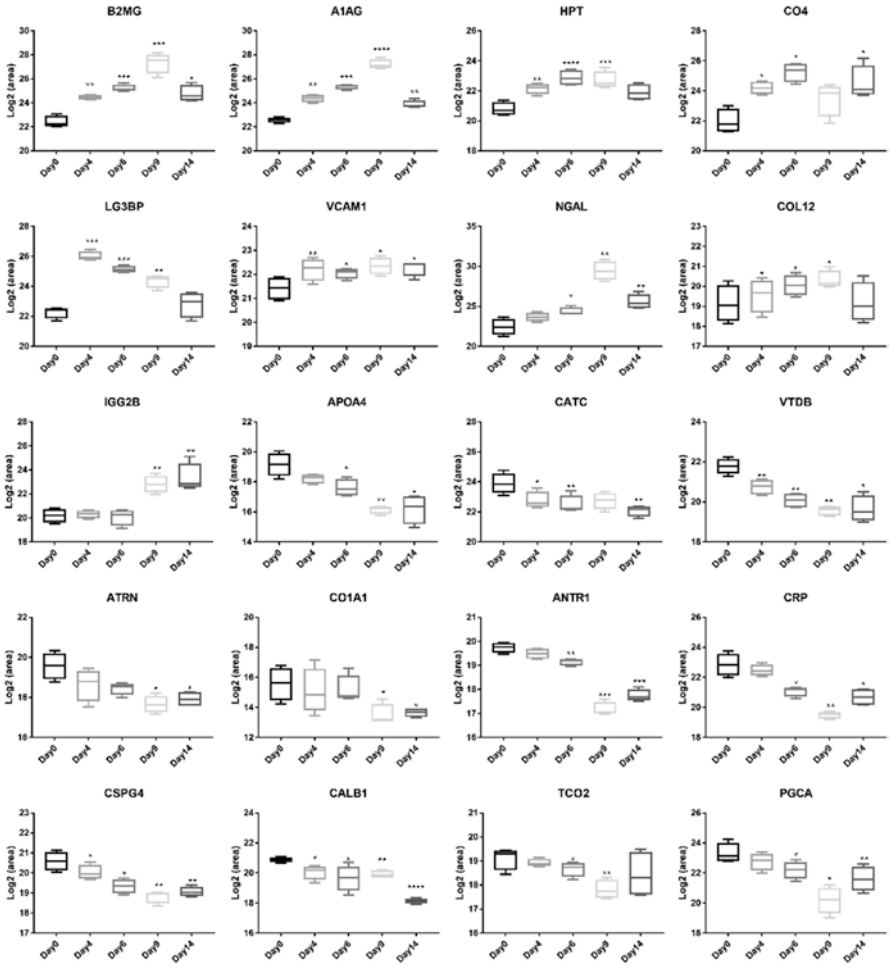

Fig. 9.3 Expression of candidate urine biomarkers during tumor development by MRM quantification. This figure is cited from (Wu et al. 2017)

quantification. Combined with label-free and targeted quantification, there were ten urinary proteins that changed significantly even before a tumor mass was palpable, including HPT, APOA4, CO4, B2MG, A1AG, CATC, VCAM1, CALB1, CSPG4, and VTDB. Moreover, these early urinary changes also showed differential abundances at late tumor stages.

In this experiment, a total of ten proteins changed significantly before a tumor mass was palpable. At this phase, the body weights of model rats were not obviously reduced, and the tumor mass might not be detected by imaging techniques. These early changed proteins continued their corresponding trends during the entire cancer

development; therefore, these urine proteins have the potential for early detection of tumors. In addition, 24 differential proteins identified in our experiment were annotated as cancer biomarkers, and some proteins have also been reported as candidate urine biomarkers in cancer patients. For example, B2MG was previously reported as a urine biomarker of several cancers (Shi et al. 2009); PDC6I was a candidate urine biomarker of the upper gastrointestinal cancer (Husi et al. 2015); urinary CO4 was reported to as a diagnostic biomarker of bladder cancer (Chen et al. 2012); many malignant tumors overexpressed urinary NGAL, which is a candidate biomarker for malignancy (Lippi et al. 2014); and KNT1 was validated as a urinary biomarker of breast cancer (Gajbhiye et al. 2016).

9.3 Changes of Urine Proteome in Other Tumor-Bearing Models

The lung tumor-bearing model was established by tail-vein injection of 2×10^6 viable W256 cells into male Wistar rats (150 ± 20 g) (Wei et al. 2018). Urine samples were collected from lung carcinoma rats on days 2, 4, 6, and 9 after W256 cell injection. Using label-free quantification, a total of 139 differential urine proteins were identified at four time points. There were 43, 34, 90, and 81 differential proteins on days 2, 4, 6, and 9 after tail-vein injection, respectively. After label-free quantitative analysis, 20 differential proteins changed on days 2 or 4 commonly identified by two mass spectrometers (Triple TOF 5600 and Orbitrap Fusion Lumos) were validated with PRM analysis. Finally, nine urine proteins were successfully validated with differential abundance consistent with label-free quantification. Among these nine differential proteins, LG3BP (Fukuda et al. 2017), A1AG (Ayyub et al. 2016), CO4 (Ajona et al. 2013), APOE (Su et al. 2011), and VTDB (Turner et al. 2013) were previously reported as disease biomarkers of lung cancer. Because lung histopathological examination showed that the lung metastasis nodules appeared on day 4, these differential urine proteins could be used for early detection of lung metastatic tumors.

The brain tumor-bearing model was established by injection of 2000 W256 tumor cells into the brain of male Wistar rats (180–200 g) (Zhang et al. 2018b). Urine samples were collected on days 3, 5, 8, and 10 after tumor cell injection. Urine samples at four time points were analyzed using an Orbitrap Fusion Lumos Tribrid mass spectrometer. A total of 102 differential proteins were identified, of which there were 21, 33, and 75 proteins differentially expressed on days 3, 5, and 8, respectively. On days 2, 5, 7, 9, and 12 after the tumor cell injection, rats were subjected to small animal MRI scans. It was observed that only a smaller tumor lesion was detected on day 9 in the right side of the brain. Thus MRI imaging did not significantly change until the 5th day after intracerebral W256 cell injection, but 43 urine proteins had shown differential abundance at this stage. These results suggested that urine proteins changed significantly at early tumor phase even before

changes in MRI imaging. Then, urine samples from another four model rats were reanalyzed using label-free quantification as before for biomarker validation. Finally, there were 28 commonly identified differential proteins in eight tumor-bearing rats. Among these proteins, LG3BP, B2MG, CSF1, A1AG, MUC18, NGAL, ICAM-1, VCAM-1, APOE, APOA-I, and FBP1 had been previously reported as biomarkers of primary brain tumor or brain metastasis.

The bone tumor-bearing model was established by injection of 100 W256 tumor cells into the humeral bone cavity of male SD rats (180–200 g) (Wang et al. 2019). Tumor growth was detected using small animal PET-CT. Urine samples were collected on days 3, 5, 7, and 13 after tumor cell injection. Using label-free quantification, a total of 69 differential proteins were identified, of which there were 25, 13, 20, and 27 differential urine proteins on days 3, 5, 7, and 13, respectively. By CT imaging, it was observed that tumor cells grew at the injection location on day 5, and bone density was significantly decreased on day 10 after W256 tumor cell inoculation. Several differential proteins identified on day 3 and day 5 were associated with cancer bone metastasis or osteoarthritis, including SPP1, GDF15, TNFRSF1B, MCAM, PLOD3, ICAM1, GLBP3, and B2MG.

The liver tumor-bearing model was established by injection of 2.5×10^5 W256 tumor cells into liver of male Wistar rats (180–200 g) (Zhang et al. 2019). Urine samples from four time points (days 3, 5, 7, and 11) were used for label-free proteome quantification, and a total of 98 differential proteins were identified. Twelve proteins on day 3 and 55 proteins on day 5 changed significantly before the obvious pathological appearance. Several urine proteins changed at early phase of disease had been reported as biomarkers of liver cancer, such as CO4, PIgR, LHPP, SBP1, ENOA, MTO, NNE, F16P1, A1AG, PRDX6, ALDOB, PRDX1, CO3, LDHB, and ICAM-1.

9.4 Comparison of Differential Proteins Among Five Tumor-Bearing Models

We compared the differential urinary proteins identified from these five tumor-bearing models. The overlap of differential proteins identified in five tumor-bearing models was shown in Fig. 9.4. It was found that many differential proteins were identified in at least two models. Interestingly, a total of ten differential urine proteins, including LG3BP, CO4, B2MG, A1AG, ABHEB, THIO, SODC, LYAG, DPP2, and ICAM1, are commonly identified in five tumor models. Additionally, it was observed that 29 differential proteins were only identified in the subcutaneous tumor model, 40 differential proteins in lung tumor model, 41 differential proteins in brain tumor model, 29 differential proteins in bone tumor model, and 23 differential proteins in liver tumor model. The results suggested that different models had different urine protein patterns when the same tumor cells grew in different organs of rats, and urine proteins have the potential to distinguish the injuries of same tumor cells grown in different organs.

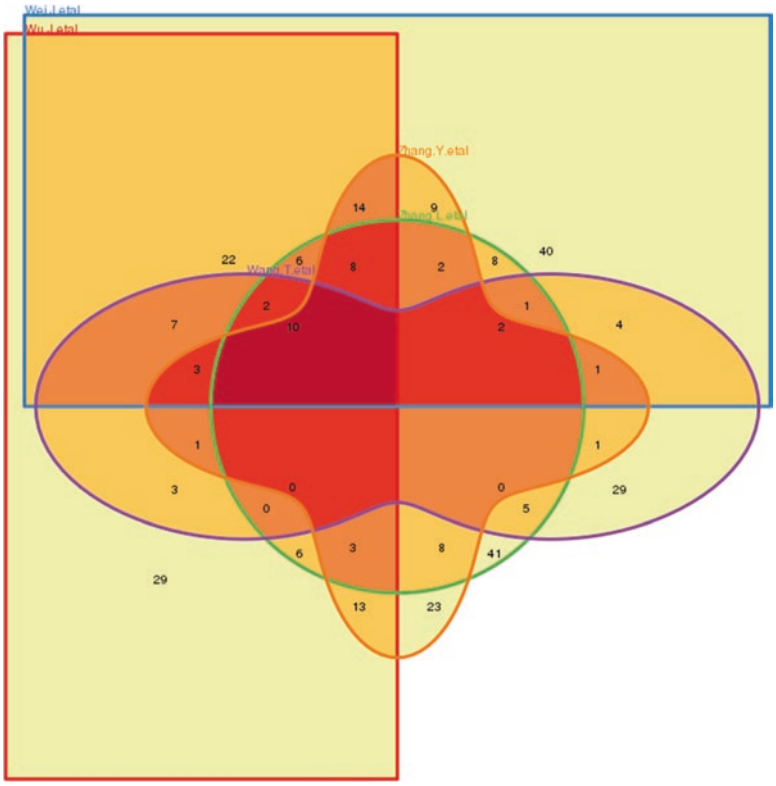

Fig. 9.4 The overlap of differential proteins identified in five different tumor-bearing models. (1) Subcutaneous tumor-bearing model by Wu et al. (2017), (2) lung tumor-bearing model by Wei et al. (2018), (3) brain tumor-bearing model by Zhang et al. (2018b), (4) bone tumor-bearing model by Wang et al. *(*2019*)*, (5) liver tumor-bearing model by Zhang et al. *(*2019*)*

9.5 Clinical Prospects

Cancer is the second leading cause of death and a major public health problem worldwide. Early diagnosis and early treatment of cancer can significantly improve survival rates for cancer patients. Early detection of cancer metastasis is also important for patient management and treatment. Therefore, noninvasive biomarkers for cancer detecting and monitoring are urgently needed, especially at early tumor stages. Urine is a promising sample source for cancer biomarker research. It can accumulate systematic changes in the body, and change is the most fundamental property of biomarkers. Moreover, urine can be noninvasively and repeatedly collected from patients. Thus, urine is suitable for monitoring cancer progression, assessing treatment response, and predicting cancer recurrence.

In tumor-bearing models, urine proteome changed significantly with tumor progression. Moreover, urine proteins could indicate the cancer presence at early tumor

growth. Of note, it is not sure whether changes of these urine proteins are specific products expressed by tumor cells or the reaction of the body to the presence of tumor. In addition, because it may be difficult to provide an accurate diagnosis for a single biomarker, a panel of urine proteins would be more sensitive and specific for cancer detection. In future studies, more urinary biomarker researches should be performed in clinical samples to evaluate the sensitivity and specificity of urine proteins for early detection of cancer. Additionally, urine protein biomarkers also have potential clinical applications in monitoring cancer treatment and prevention studies.

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Chapter 10
Candidate Urinary Biomarker Discovery in Gliomas

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Abstract Gliomas are the most common primary malignant brain tumors and have a very poor prognosis. Urine, which is not controlled by homeostatic mechanisms, can accumulate changes in the body and therefore is an ideal source for biomarker discovery. In this chapter, urine samples of animal models and glioma patients were investigated to identify candidate biomarkers of gliomas using proteomics analysis. The animal model was induced by injection of C6 cells in rat brain. Then rat urine samples were collected on days 2, 6, 10, and 13 after C6 cell inoculation. Urine samples from glioma patients were collected before and after tumor resection. Our results suggested that urinary proteins have potential as early sensitive biomarkers for detection and monitoring gliomas.

Keywords Gliomas · Urine · Proteomics · Cancer biomarkers · Early detection

10.1 Introduction

Gliomas are the most common primary malignant brain tumors, accounting for more than 60% of all brain tumors (Wang and Bettegowda 2015). Gliomas are subdivided into astrocytoma, oligodendroglioma, and oligoastrocytoma based on cell origin, and malignancy grades are assigned according to WHO criteria (Louis et al. 2007). Current therapies can only extend the survival time of glioma patients for 12–18 months with a high risk of recurrence. Early detection and recurrence monitoring are therefore essential to improve patient survival. However, no generally accepted screening protocols to reveal asymptomatic brain tumors are currently available.

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Changes associated with pathophysiological processes are the most fundamental feature of disease biomarker. Without homeostatic control, urine can accumulate changes in the body to serve as an ideal source for biomarker discovery (Gao 2013; Wu and Gao 2015). But urine may be affected by several factors, such as gender, age, exercise, and hormone conditions. Thus, it will be difficult to determine whether potential biomarkers we identified are truly related to the disease condition. To minimize the effects of confounding factors, animal models can be used to mimic the pathological process of diseases. And in clinical samples, a self-controlled study before and after glioma resection can be used to limit the interference from individual differences among patients. In this chapter, urine samples of C6 model rats at three time points and urine samples of glioma patients before and after tumor resection were used for candidate biomarker identification using comparative proteomics analysis.

10.2 Changes of Urine Proteome in Animal Model

In this study (Ni et al. 2018), 1×10^6 C6 cells were inoculated into the brain of male Wistar rats to establish the glioma model, which has similar histological characteristics of glioblastoma multiforme (GBM) (Grobben et al. 2002). GBMs are the most common and highest malignant gliomas in adults, with a median survival of only 14.6 months (Ohgaki and Kleihues 2005). In our animal experiment, magnetic resonance imaging (MRI) was performed at three time points after tumor cell inoculation. Using imaging scans, no obvious lesion was observed on day 6 after C6 cell inoculation. But on day 10, a strong enhancement with a boundary was observed in the rat brain. The strong enhancement lesions became larger on day 13, and the midline of brain was even shifted, showing an obvious intracranial placeholder (Fig. 10.1). H&E staining of brain tissue revealed that tumor tissue exhibited invasive growth. After urine proteins were extracted and digested, peptide samples were analyzed with label-free proteome quantification using an Orbitrap Fusion Lumos Tribrid mass spectrometer. A total of 778 urinary proteins were identified, and 124 differential proteins were screened at 4 time points with a fold change ≥ 2 and p value < 0.05. And 56 differentially changed urine proteins were identified on the 2nd day, 65 proteins on the 6th day, 61 proteins on the 10th day, and 27 proteins on the 13th day, respectively.

Among these differential urine proteins, 27 proteins had been reported to be associated with gliomas in brain tissue, cerebrospinal fluid (CSF), or blood. For example, NGAL complex (MMP9/NGAL) activity is elevated in gliomas (Liu et al. 2015), and it may also be involved in glioma drug resistance and clinical prognosis (Zheng et al. 2009). CNTF receptor (CNTFR) subunit alpha is involved in the formation of tumor-initiating cells, and it is also a biomarker of tumor grade in gliomas (Lu et al. 2012). Haptoglobin is involved in infection, tumor growth, and migration, and it is a candidate serum biomarker of GBM (Kumar et al. 2010). Alpha-1-acid glycoprotein is a prognostic biomarker of GBM with differential abundance in the

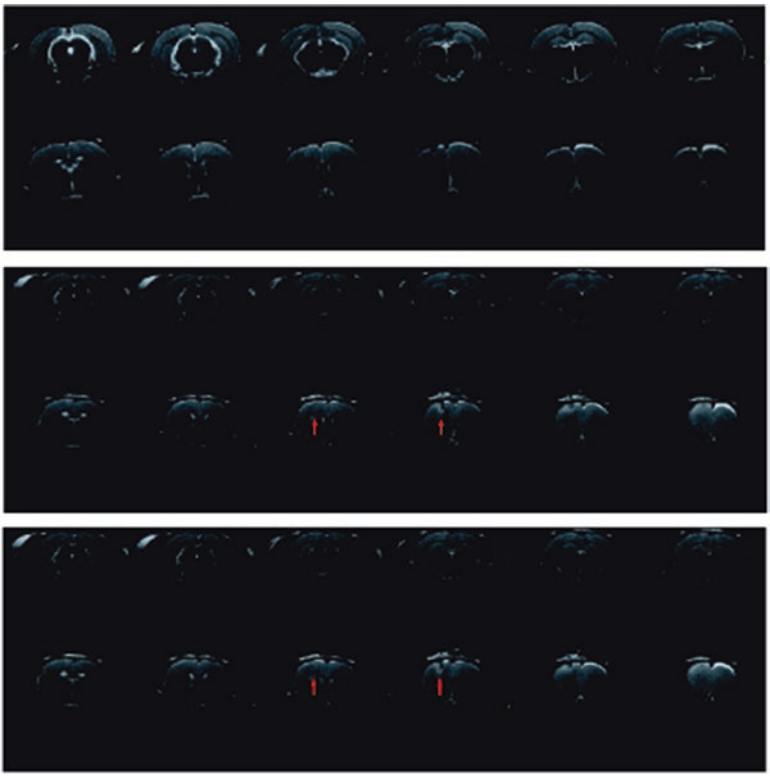

Fig. 10.1 MRI results of the brain tissues in model rats on the 6th, 10th, and 13th day after an injection with tumor cells. The red arrow indicates the cancer tissues. (This figure is cited from Ni et al. 2018)

serum (Matsuura and Nakazawa 1985). Cathepsin D (CTSD) is a lysosomal marker and is involved in autophagy and glioma invasion (Giatromanolaki et al. 2014; Pei et al. 2014). Because these differential urine proteins were identified at the early stage of tumor growth, they may enable the early detection of gliomas.

A total of 39 differential proteins were further selected for validation in other GBM rats using multiple reaction monitoring (MRM) assay. After validation, 34 urinary proteins were downregulated, and 5 proteins were upregulated. In Table 10.1, we listed some important urine proteins identified on days 2 and 6, which were validated by MRM. These early changed urine proteins have the potential for the non-invasive diagnosis of GBM. But it should be noted that some proteins only significantly changed on the 2nd day after tumor cell inoculation, but their expression recovered to normal levels at later time points. It does not rule out one possibility that changes of these proteins were the response to stress caused by tumor cells in the brain. In addition, some differential proteins were also annotated as urine biomarkers in Urinary Protein Biomarker database (Shao et al. 2011), which means they were also identified with differential abundance in the urine of other diseases.

Table 10.1 Differential urine proteins identified on the 2nd and 6th day in GBM rats

UniProt number	Protein name	Trend	Fold change				Associate with GBM	Urine biomarkers
			D2	D6	D10	D13		
P80188	Neutrophil gelatinase-associated lipocalin	↑	7.64	–	–	–	Tissue (Liu et al. 2011)	Yes
P08294	Extracellular superoxide dismutase [cu-Zn]	↑	2.60	–	–	–	–	Yes
P00738	Haptoglobin	↑	4.58	–	–	–	Blood (Kumar et al. 2010)	–
P02763	Alpha-1-acid glycoprotein	↑	7.33	–	–	–	Blood (Matsuura and Nakazawa 1985)	Yes
Q06830	Peroxiredoxin-1	↓	–	0.34	–	–	Tissue (Martelli et al. 2015)	–
P68032	Actin, alpha cardiac muscle 1	↓	–	0.46	–	–	Tissue (Ohtaki et al. 2017)	–
P07339	Cathepsin D	↓	–	0.47	–	–	Tumor cells (Giatromanolaki et al. 2014)	–
P15311	Ezrin	↓	–	0.46	–	–	Tissue (Tynninen et al. 2004)	–
P23528	Cofilin-1	↓	–	0.40	–	–	Tissue (Yan et al. 2012)	–
P15104	Glutamine synthetase	↓	0.47	0.42	–	–	Tumor cells (He et al. 2016)	–
Q14894	Ketimine reductase mu-crystallin	↓	0.44	0.46	–	–	–	–
O95154	Aflatoxin B1 aldehyde reductase member 3	↓	0.25	0.27	–	–	–	–
O43707	Alpha-actinin-4	↓	0.04	0.15	–	–	Tissue (Quick and Skalli 2010)	–
P02741	C-reactive protein	↓	–	0.39	0.42	–	Blood (Nijaguna et al. 2015)	Yes
P62158	Calmodulin	↓	–	0.42	0.49	–	–	–
P51674	Neuronal membrane glycoprotein M6-a	↓	–	0.27	0.40	–	–	–
P01024	Complement C3	↓	–	0.40	–	0.45	Tissue (Cheng et al. 2011)	–
P15144	Aminopeptidase N	↓	0.33	0.30	0.46	–	Tissue (Ramirez-Exposito et al. 2014)	–
P48637	Glutathione synthetase	↓	0.44	0.20	0.31	–	Tumor cells (Han et al. 2015)	–
Q15493	Regucalcin	↓	0.28	0.18	0.33	–	–	–
P46721	Solute carrier organic anion transporter family member 1A1	↓	0.07	0.08	0.23	–	–	–

P60709	Actin, cytoplasmic 1	↓	0.49	0.32	0.44	–	–	–
Q9Y696	Chloride intracellular channel protein 4	↓	0.33	0.25	0.41	–	–	–
P09211	Glutathione S-transferase P	↓	0.37	0.22	0.28	–	Tissue (Stavrinou et al. 2015)	Yes
Q9UHI7	Solute carrier family 23 member1	↓	0.25	0.18	0.20	–	–	–
P08236	Beta-glucuronidase	↓	0.11	0.08	0.13	–	Tissue (Bensalma et al. 2015)	–
O00299	Chloride intracellular channel protein 1	↓	0.45	0.34	0.49	–	Tissue (Setti et al. 2015)	–
O14745	Na (+)/H (+) exchange regulatory cofactor	↓	0.38	0.28	0.37	–	–	–
Q9UNQ0	ATP-binding cassette sub-family G member 2	↓	0.32	0.27	0.41	–	–	–
P08473	Neprilysin	↓	–	0.30	0.43	0.46	Tissue (Monod et al. 1992)	Yes
Q03154	Aminoacylase-1A	↓	–	0.48	0.41	0.49	–	–
P05186	Alkaline phosphatase	↓	0.23	–	0.33	0.37	Tissue (Iwadate et al. 2016)	Yes
Q07075	Glutamyl aminopeptidase	↓	0.36	0.25	0.27	0.38	–	–
Q8TCU3	Solute carrier family 7 member13	↓	0.16	0.13	0.21	0.43	–	–
Q9H4M9	EH domain-containing protein 1	↓	0.13	0.19	0.27	0.46	–	–

Therefore, it will be difficult to provide an accurate diagnosis of gliomas based on a single biomarker, and a panel of urine proteins would improve the sensitivity and specificity of glioma diagnosis. In this experiment, MRI imaging changes appeared on day 10 after model establishment, whereas changes of urine proteins can provide early valuable clues from day 2 or day 6. Among those differential proteins identified at this early stage, some proteins were previously reported as candidate biomarkers of GBM, suggesting that urine proteins may be an excellent choice for the early detection of gliomas.

10.3 Changes in Urine Proteomes of Glioma Patients Before and After Tumor Resection

In this study (Wu et al. 2018), urine samples from five glioma patients (1 WHO II; 4 WHO IV) were collected at the time of tumor diagnosis and after surgical removal of brain tumors. Postoperative urine samples were collected 1–2 weeks after tumor resection to avoid stress from trauma affecting the urine proteome. An image of a representative patient brain before and after tumor resection is shown in Fig. 10.2. A comparative proteomics analysis of urine samples before and after tumor resection was performed using LC-MS/MS. The first proteome analysis was performed with a 60-min running time on an Orbitrap Fusion Lumos Tribrid mass spectrometer. A total of 1377 urinary proteins were identified with ≥ 2 unique peptides and a FDR $< 1\%$ at protein level. And 27 differential urine proteins were identified, of which 20 proteins can be detectable in human glioma tissues based on the Human Protein Atlas database. After functional annotation of these differential proteins, it was found that some biological processes, such as regulation of tissue remodeling, autophagy, negative regulation of gene expression, and angiogenesis, were overrepresented. The pathways for the lysosome, the renin-angiotensin system, and the phagosome were significantly enriched.

To identify more urinary proteins and validate the differential proteins identified in the first analysis, ten samples were reanalyzed using a 90-min running time. In

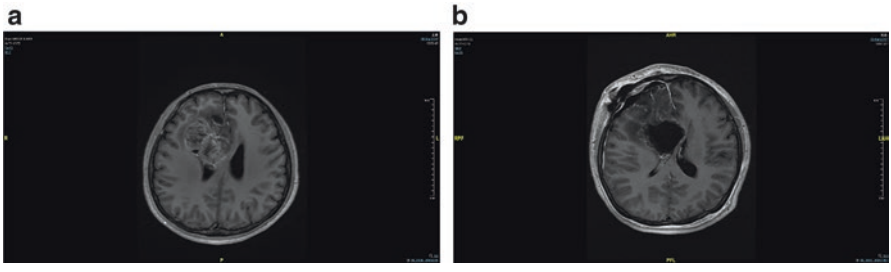

Fig. 10.2 MRI of the brain before and after tumor resection. (**a**) MRI for the tumor and (**b**) after tumor removal. (This figure is cited from Wu et al. 2018)

the second analysis, 1652 urinary proteins were identified. Moreover, a total of 17 differential proteins were commonly identified in both proteomic analyses.

Similarly, some differential urine proteins identified in clinical samples are associated with gliomas, such as CEACAM1, ANXA7, CALR, CRYAB, CD276, PIGR, CTSD, ASAH1, and beta-glucuronidase. For example, silencing of CEACAM1 can promote apoptosis in human glioma cells, and it is a potential therapeutic target for gliomas (Xu et al. 2015). ANXA7 protein expression is decreased in glioma tissues, and its degradation is associated with glioma progression and prognosis (Hung and Howng 2003; Pan et al. 2015). CALR downregulated in glioma tissues and its expression is correlated with tumor grade and patient survival (Gao et al. 2013). CRYAB upregulated in GBM and its knockdown can increase the invasiveness of glioma cells (Kore and Abraham 2014; Shimizu et al. 2016). CD276 upregulated in high-grade glioma and its expression correlates with malignancy grade and patient survival (Zhou et al. 2013; Lemke et al. 2012). PIGR was reported as a prognostic predictor after tumor resection in glioma patients (Niu et al. 2014). ASAH1 is a drug target for pediatric brain tumors (Doan et al. 2017). Beta-glucuronidase is an enzyme found in the necrotic areas of GBM (Bensalma et al. 2015). Thus, these urine proteins have the potential clinical applications for detecting cancer presence and monitoring cancer recurrence after treatment in glioma patients. As low-grade gliomas have a relative longer survival time, recurrence monitoring is more necessary in these patients.

10.4 Clinical Prospects

In recent years, some tumor-associated circulating biomarkers have been identified in blood and cerebrospinal fluid (CSF) of glioma patients (Kros et al. 2015). Potential protein biomarkers in CSF include OPN, IGFBP-2, MIF, AACT, TTHY, ALB, gelsolin, attractin, VEGF, FGF-ß, L-CaD, CEA, PDGF, CD95, AFP, and HCG. Potential protein biomarkers in blood include VEGF, VEGFR2, EGFR, PlGF, FGF-ß, OPN, Agn2, Tie2, IGFBP-2, galectin-1, IGFBP-2, IGFBP-5, YKL-40, MMP2, MMP9, MMP10, Ang-2, AHSG, PBEF1, PAI-1, GFAP, NCAM, eNOS, APRIL, L-CaD, cathepsin D, recoverin, CEA, PDGF, AFP, and HCG. Compared with CSF and blood, urine has been severely ignored in biomarker studies for gliomas. However, a few available urine-based studies have suggested that urine is a good source for relevant biomarkers of brain diseases (Smith et al. 2008; An and Gao 2015).

Interestingly, in our study most differential urine proteins were downregulated with cancer presence in both animal model and clinical samples. Maybe these proteins are highly needed by tumor tissue to participate in tumorigenesis, and brain tissue no longer releases them in circulation. In this condition, levels of these proteins may increase in the glioma tissues but decrease in the urine. Another possibility is that tumor tissue of gliomas may inhibit the expression of these proteins. Then their expression both decreased in the brain and urine. However, further experi-

ments are needed to validate this hypothesis. In addition, five differential urine proteins are commonly identified in the C6 rat glioma model and clinical samples, including dihydropteridine reductase, glutamyl aminopeptidase, aminopeptidase N, beta-glucuronidase, and CTSD, suggesting animal model and clinical patients have some similar pathophysiological processes.

In the present study, despite the preliminary results and small number of samples, we identified some biomarker candidates associated with gliomas. Our results show great promise of urine biomarker for monitoring gliomas or other brain cancers. In future studies, we hope our work will help to understand the role of urine as a promising sample source in biomarker discovery of brain diseases. A larger number of clinical samples are also needed to validate specific urinary protein panels for clinical application as noninvasive biomarkers for early glioma detection and/or monitoring glioma recurrence.

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Chapter 11
Changes in the Urinary Proteome in a Patient-Derived Xenograft (PDX) Nude Mouse Model of Colorectal Tumor

Yongtao Liu

Abstract In this report, the urinary proteome from a patient-derived xenograft (PDX) model was examined at the peptide level to study the origins of urinary proteins in tumor-bearing nude mice. Urine was collected from PDX mice before and after colorectal tumor implantation. A total of 4318 unique peptides were identified, and 78 unambiguous human-origin peptides were discerned in the PDX model urine. Unlike the differential urinary protein composition of tumor-bearing immunocompetent rat models, the differential urinary proteins in the PDX model did not include host immune response proteins. This study demonstrates that tumor-secreted proteins can be observed in the urine proteome of the PDX model. However, immune response proteins, which are very early candidate tumor biomarkers, are not present in the urine of PDX model mice; this absence is due to immune deficiency. Therefore, immunodeficient animals may not be suitable models for searching for early immunity-associated tumor biomarkers in the urine.

Keywords Proteomics · PDX model · Urine · Cancer biomarkers

11.1 Introduction

Biomarkers are measurable changes associated with physiological or pathophysiological processes. Whereas blood remains stable and balanced due to homeostatic mechanisms, urine, which is where most blood wastes are disposed, exhibits substantial changes (Gao 2013).

The urinary proteome of rats subcutaneously injected with the Walker 256 tumor cell line has been shown to change significantly before a tumor mass is palpable. Some of the involved proteins have been reported as tumor markers or are associated with vaccine-related tumors (Wu et al. 2017). Similarly, changes in the urinary proteome of rats injected with C6 glioma cells in the brain were observed before the tumors could be detected by magnetic resonance imaging (MRI). Many of these

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differential urinary proteins were previously reported to be associated with glioma (Ni et al. 2018).

Since differential urinary proteins are present in the very early stages of tumor cell implantation, it is unlikely that the substantial changes in the urine result from a small number of tumor cells. Therefore, we investigated whether human proteins could be found in the urine of tumor-bearing nude mice. In the patient-derived xenograft (PDX) model (Gao 2016), human-origin tumors can grow due to the absence of normal T cell immunity. Human-origin peptides that are unambiguously identified in the urine of the model must originate from the human tumor. If mouse urinary proteins also change, then information about the pathophysiological changes in the host mouse may be reflected in these changes.

In this study, colorectal tumor tissues from patients were implanted subcutaneously into nude mice to establish a PDX model. Urine samples were collected from the nude mice before and after tumor transplantation. The samples were analyzed using bottom-up proteome analysis, and the urinary proteins were digested in gel and profiled by liquid chromatography tandem mass spectrometry (LC-MS/MS). The identified peptides were compared at the peptide level (Yin et al. 2014).

11.2 Tumor Acquisition and PDX Model Establishment

The human tumor tissues used in this study were collected from surgical patients at Peking Union Medical College Hospital. All patients fully understood and signed informed consent forms. Pathogen-free, 8–10-week-old female NOD SCID mice and 10–12-week-old female BALB/c nude mice were purchased from Beijing Vital River Laboratory Animal Technology Co., Ltd. All animals were housed under temperature-controlled conditions with proper humidity, lighting (12 h light/12 h dark cycle), and free access to food and water. Fresh tumor samples at least $6 \times 6 \times 6$ mm (> 200 mm^3) in size were obtained in situ or from metastatic colorectal tumor tissue after surgery. The sampling sites showed highly malignant tissue activity. After collection, the samples were repeatedly rinsed with precooled sterile saline and immediately placed in precooled specialized preservative solution. The patient tumor tissues were placed individually into plates containing RPMI 1640 medium (Gibco, Paisley, UK) and transported to the laboratory at 4 °C. The tumor tissues were cleaned, and connective tissue, blood vessels, adipose tissue, and regions of calcification and necrosis were removed from the surface. Then, each tumor tissue was cut to obtain a 3–5 mm^3 tumor mass. The tumor masses were implanted at 4 subcutaneous points in each of two female NOD/SCID mice. Tumor growth was observed daily. When the tumors reached a threshold volume, the tumor-bearing mice were sacrificed, and the tumors were dissected. Next, the tumors were cleaned, cut into small pieces of $3 \times 3 \times 3$ mm, and inoculated subcutaneously into 20 female BALB/c nude mice (Tentler et al. 2012; Hidalgo et al. 2014; Siolas and Hannon 2013). Each mouse was inoculated at 1 point. After 23 days, when the tumor volume had reached 300 mm^3, urine was collected from the BALB/c nude mice.

11.3 Urine Collection and Sample Preparation

Animals were placed individually in metabolic cages overnight (for 12 h) to collect urine samples. During urine collection, the mice had free access to water but not food to avoid urine contamination. The collected urine was centrifuged at 4 °C and 3000 × g for 10 min to remove cells and particulate matter and then stored at −80 °C. Centrifugation of the samples at 4 °C and 12,000 × g for 30 min was performed to remove cell debris before urinary protein extraction. The supernatants were precipitated with three volumes of acetone precooled at −20 °C for 2 h, followed by centrifugation at 4 °C and 12,000 × g for 30 min. Then, the precipitate was resuspended in lysis buffer (8 mol/L urea, 2 mol/L thiourea, 25 mmol/L DTT, and 50 mmol/L Tris (Sigma-Aldrich, Germany) (Sun et al. 2005). The protein concentrations were measured using the Bradford assay at 595 nm. An albumin standard (Thermo Fisher, USA) was used [bovine serum albumin (BSA) at 2 mg/mL in 0.9% saline and 0.05% sodium azide].

11.4 Tryptic Digestion in Gels

Before each tumor-bearing nude mouse was inoculated with the tumor, urine was collected as a control. Urine samples from eight mice from each of the control and tumor-bearing groups were collected and analyzed by mass spectrometry with in-gel digestion. Eighty micrograms of total protein were mixed with 5 × loading buffer. The proteins were completely denatured at 95 °C for 10 min and loaded into 4–12% Bis-Tris preformed protein gels (Life, NuPAGE) in 1 × MES SDS running buffer (Life, NuPAGE). Then, the proteins were separated by electrophoresis at 200 V for 40 min. The gels were dyed with Coomassie blue for a few minutes to allow easier fading. Each gel was decolorized 2–3 times in a solution containing 30% methanol and 10% acetic acid until the gel background had lost as much of the blue stain as possible. The entire process was carried out in a container, and the same chemical reagents and processing time were used for all the gels to ensure the results of multiple gels could be reliably compared. Each lane was cut from the bottom-up into five pieces based on the concentration in the gel, and each section was cut into 1 –1.5 mm^3 pieces. The pieces were washed with 25 mmol/L ammonium bicarbonate/acetonitrile solution (1:1 V/V) until they were thoroughly discolored. Dithiothreitol (DTT) was applied at 20 mmol/L and 37 °C for 1 h to denature the disulfide bonds in the protein structure, and 55 mmol/L iodoacetamide (IAM) was added for 30 min in the dark to alkylate the disulfide bound sites. Next, 5 μg/L of trypsin (Trypsin Gold, Promega, Fitchburg, WI, USA) was added to the dried gel pieces, which were then incubated at 37 °C overnight. Peptides were collected with 50% acetonitrile; then, the process was repeated, and the proteins were lyophilized and stored at −80 °C.

11.5 Nano LC-MS/MS Analysis

The reconstituted peptides were desalted with a C18 Zip-Tip (Millipore, Germany) and dissolved in 0.1% formic acid. LC-MS/MS analysis was performed using an EASY-nLC 1200 system coupled to an Orbitrap Fusion Lumos mass spectrometer (Thermo Scientific). The liquid chromatography parameters were as follows: precolumn, 75 μm × 2 cm, nanoViper C18, 2 μm, and 100 Å (Thermo Fisher Scientific, USA); analytical column, 50 μm × 15 cm, nanoViper C18, 2 μm, and 100 Å; and injection volume, 2 μL. The flow rate was 250 nL/min. Phase A was 0.1% formic acid/water (Fisher Scientific, Spain), and phase B was 80% acetonitrile (Fisher Chemical, USA)/0.1% formic acid/20% water. The ion origin was nano-ESI, and MS1 data were collected by Orbitrap with a resolution of 120,000, an ion charge range of 2–7, and high-energy collisional dissociation (HCD) by applying a normalized collision energy of 32%. MS2 data were collected by Orbitrap at a resolution of 30,000. The HeLa Protein Digest Standard (Thermo Scientific, 88,329) was used to evaluate instrument performance.

11.6 Data Analysis and Bioinformatics Analysis

The raw MS data were processed using the Proteome Discoverer platform (Thermo Scientific, version 2.1) and the Sequest HT algorithm. Proteomic data were searched using the UniProt *Homo sapiens* and *Mus musculus* databases (updated Sep 2017). The search parameters were set as follows: the MS deviation of the peptide precursor and product ions was 0.05 Da; variable modifications included oxidation (M) and protein N-terminal acetylation and deamidation (N and Q); fixed modification included carbamidomethylation (C); and two missing trypsin cleavage sites were allowed. The peptide false discovery rate (FDR) was less than 1%.

Urine sample information for the nude mice was obtained from the online free-ware DAVID Bioinformatics Resources 6.8 (https://david.ncifcrf.gov) for functional annotation assessment, including annotation of protein molecular functions, cell components, and biological processes (The overall process is shown in Fig. 11.1).

11.7 Urinary Proteome Profiling at the Peptide Level

After processing the data with Proteome Discoverer 2.1 and the Sequest HT algorithm, the MS data (.raw files) were searched against the *Homo sapiens* and *Mus musculus* databases to obtain peptide sequences and protein descriptions.

In total, 4318 unique peptides were detected from the samples from the control and tumor-bearing groups. After manually comparing the homologous peptide sequences of mouse and human at the same protein locations (sequence data from

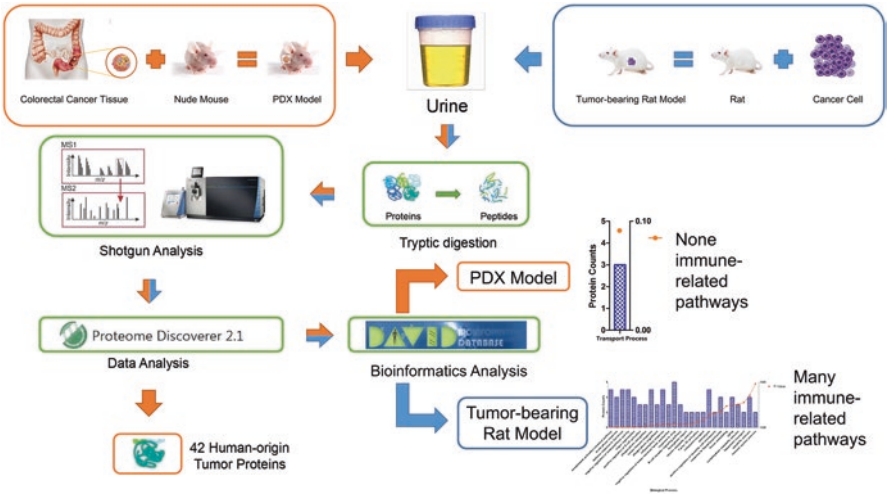

Fig. 11.1 Flow chart of this experiment. Colorectal tumor tissues from patients are implanted subcutaneously into nude mice to establish the PDX model. Urine samples are collected from the nude mice before and after tumor transplantation. The samples are analyzed using bottom-up proteome analysis, and the urinary proteins are digested in gel and profiled by LC-MS/MS. The identified peptides are compared at the peptide level

UniProt, https://www.uniprot.org/), more than 3800 peptides with same sequence for both human and mouse were removed. Seventy-eight unambiguous human peptides from 42 human proteins were identified with certainty among the 4318 peptides, and each of these 78 peptides was confirmed on the MS2 spectrum (Fig. 11.2). The 78 peptides/42 human proteins were present only in the tumor-bearing mice; they were not detected in the control mice. The confidence of the peptide spectrum matches (PSMs) was greater than 99%, which was filtered by a 1% FDR. Figure 11.3 shows the main flow of the data analysis.

11.8 Unambiguous Human Peptides and Proteins

In the PDX model, 42 unambiguous human-origin proteins (corresponding to 78 human-specific peptides) were found in the urine on day 23 after tumor transplantation. These proteins must have originated from the human tumor.

Each human-origin protein was searched in the human urinary protein database (https://www.urimarker.com/urine/) (Zhao et al. 2016) (Fernandez-Olavarria et al. 2016), which contains information on nearly 6000 normal human urinary proteins. This database is available online and contains the most comprehensive information available on human urinary protein biomarkers. By searching this database, the abundance of each tumor-secreted protein in normal human urine was determined. The results are shown in Table 11.1.

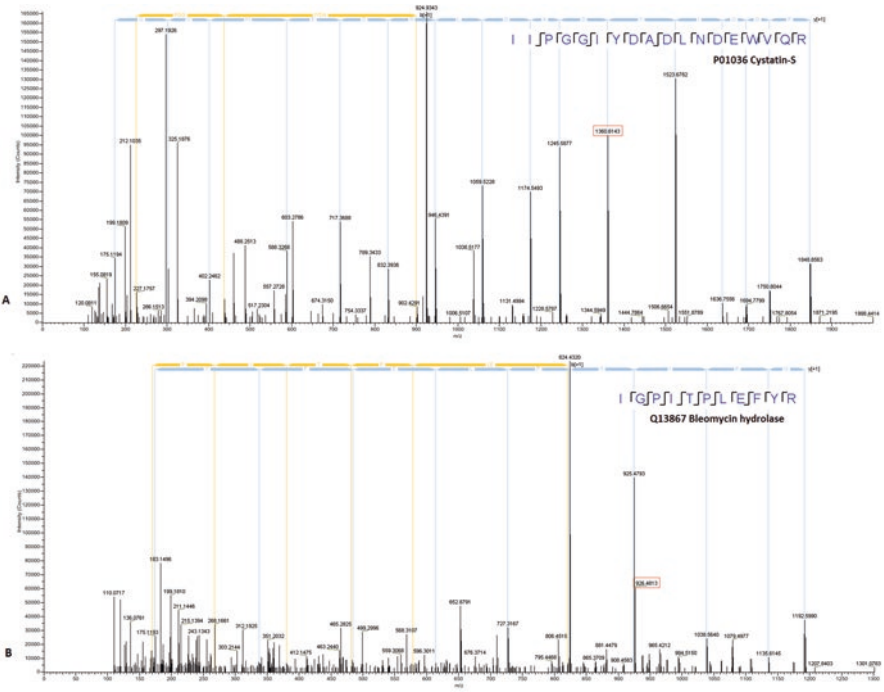

Fig. 11.2 Partial displays of the MS2 of 78 human peptides. The tandem mass spectrometry fragmented the precursor ions of the peptides by HCD and produced different b + and y + product ion pairs. The peptide sequence information was obtained by calculating the mass-to-charge ratio (m/z) of adjacent fragment ions and matching with the database to obtain the species and protein descriptions of the peptides. Human peptides found by MS2 spectrum sequence associated with cystatin-s (**a**) and bleomycin hydrolase (**b**) are shown. The total spectrum is provided in Supplementary Information

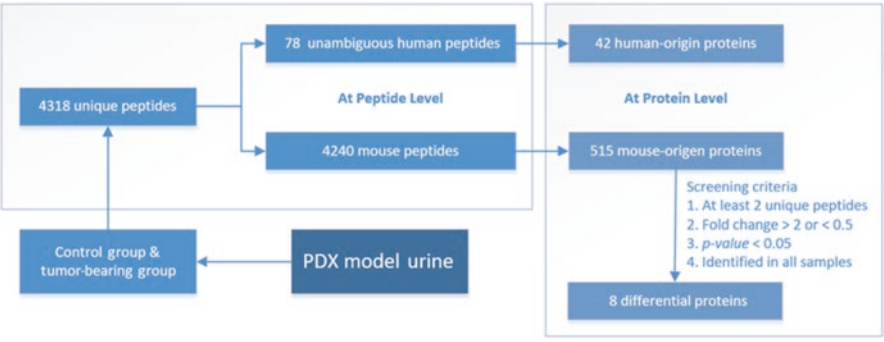

Fig. 11.3 The main flow of data analysis. First, the species origin of the proteins was identified at the peptide level, and then the results were searched for human-origin unambiguous peptides. Subsequently, analysis was performed at the protein level. The peptide-level results were converted to protein-level results for the screening and comparison of differential proteins

Table 11.1 Human-origin tumor peptides and protein information from the tumor-bearing nude mice

Accession	Protein description	Identification counts	Unique peptide counts	Concentration in human urine (pg/mL)
B4DV14	Highly similar to Napsin-A	69(4/4)	1	Not found
A1A508	PRSS3 protein (PRSS3)	44(1/4)	1	Not found
D9YZU5	Beta-globin (HBB)	33(1/4)	6	73,156.39
P99999	Cytochrome c (CYCS)	26(3/4)	4	235.11
A8K7G6	Highly similar to *Homo sapiens* regenerating islet-derived 1 alpha	22(1/4)	3	Not found
A0A087WXI5	Cadherin-1 (CDH1)	17(3/4)	4	61,234.54
P48304	Lithostathine-1-beta (REG1B)	14(1/4)	1	Not found
A0A0A6YYJ4	Trefoil factor 3 (TFF3)	13(2/4)	4	13,032.49
P04083	Annexin A1 (ANXA1)	12(1/4)	1	25,421.29
P01037	Cystatin-SN (CST1) (Zaman et al. 2018)	11(1/4)	5	3490.18
A0A024RAM2	Glutaredoxin (thioltransferase) (GLRX)	9(2/4)	1	Not found
P01036	Cystatin-S (CST4)	7(1/4)	1	4655.98
A0A1K0GXZ1	Globin C1 (GLNC1)	7(1/4)	2	Not found
S6B294	IgG L chain	7(1/4)	1	Not found
H9ZYJ2	Thioredoxin (TXN)	7(2/4)	2	Not found
P36957	Dihydrolipoyllysine (DLST)	6(1/4)	1	4061.93
V9HWA9	Epididymis secretory sperm binding protein li 62p (HEL-S-62p)	6(1/4)	2	Not found
Q8TAX7	Mucin-7 (MUC7)	6(1/4)	3	Not found
Q6N092	DKFZp686K18196	6(2/4)	3	Not found
Q99988	Growth/differentiation factor 15 (GDF15)	4(2/4)	2	3499.81
A0A1U9X8X6	CDSN	3(1/4)	1	Not found
P04406	Glyceraldehyde-3-phosphate dehydrogenase (GAPDH)	3(1/4)	1	20,395.34
P31151	Protein S100-A7 (S100A7)	3(2/4)	2	1600.63
Q96DA0	Zymogen granule protein 16 homolog B (ZG16B)	3(1/4)	2	14,970.59
Q8N4F0	BPI fold-containing family B member 2 (BPIFB2)	2(1/4)	2	752.72
A9UFC0	Caspase 14 (CASP14)	2(1/4)	2	Not found
Q76LA1	CSTB	2(1/4)	2	Not found
P01040	Cystatin-A (CSTA)	2(1/4)	2	1309.68
Q05DB4	HEBP2	2(1/4)	2	Not found
A7Y9J9	Mucin 5 AC	2(1/4)	2	Not found

(continued)

Table 11.1 (continued)

Accession	Protein description	Identification counts	Unique peptide counts	Concentration in human urine (pg/mL)
Q03403	Trefoil factor 2 (TFF2)	2(1/4)	1	59,910.81
Q13867	Bleomycin hydrolase (BLMH)	1(1/4)	1	262.38
Q8TCX0	Delta 2-isopentenyl pyrophosphate transferase-like protein	1(1/4)	1	Not found
V9HW80	Epididymis luminal protein 220 (HEL-S-70)	1(1/4)	1	Not found
B7Z3K9	Fructose-bisphosphate aldolase	1(1/4)	1	Not found
Q6FH62	HSD17B3	1(1/4)	1	Not found
X6R7Y7	Intraflagellar transport protein 25 homolog (HSPB11)	1(1/4)	1	Not found
Q96P63–2	Serpin B12 (SERPINB12)	1(1/4)	1	Not found
P59665	Neutrophil defensin 1 (DEFA1)	1(1/4)	1	20,375.74
P01833	Polymeric immunoglobulin receptor (PIGR)	1(1/4)	1	129,844.97
A0A158RFU6	RAB7	1(1/4)	1	Not found
P29508	Serpin B3 (SERPINB3)	1(1/4)	1	26,103.15

Note: The contents in parentheses after "identification counts" are expressed as the number of animals/total number of animals per group

Of these 42 human proteins, 21 have been reported in normal human urine, including 16 high-abundance proteins (concentrations greater than 1000 pg/mL), 3 moderate-abundance proteins (concentrations greater than 100 pg/mL), and 2 low-abundance proteins (concentrations less than 100 pg/mL); the remaining 21 proteins did not appear in normal human urine. Half of the 42 human tumor proteins have been detected in normal human urine. This finding indicated that these proteins, derived from tumor cells in this study, are found in normal human urine (Emmink et al. 2013; Nandy and Seal 2016). Table 11.1 provides information on the 42 human proteins, 78 specific peptides (see Supplementary Dataset 1), and their concentrations in normal human urine as determined by database search. Identification PSM counts are shown in the third column, and the ratio of the number of tumor-bearing mice in which the peptides were identified to the total mice number is presented in parentheses.

The biological process analysis of the 42 human-origin proteins (Fig. 11.4a) revealed that numerous biological processes were related to digestion and hydrolysis of peptides or proteins (negative regulation of proteolysis, negative regulation of peptidase activity, negative regulation of endopeptidase activity, digestion, and

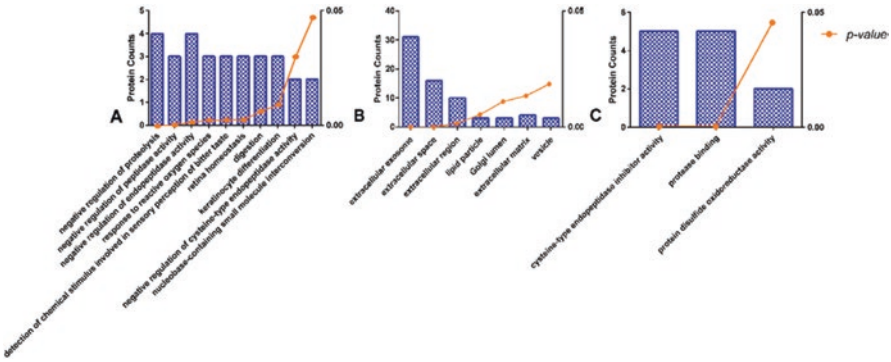

Fig. 11.4 Functional analysis of human-origin tumor proteins in tumor-bearing nude mice. (**a**) Biological process, (**b**) cellular component, and (**c**) molecular function. The ordinate on the left is the protein counts in the relevant pathway and on the right is the *p-value* of the relevant pathway. A smaller *p-value* indicates a more significant relationship between the pathway and proteins

negative regulation of cysteine-type endopeptidase activity) and response to reactive oxygen species. In the cell composition analysis (Fig. 11.4b), most of the 42 human-origin proteins were derived from the secretion of extracellular exosomes or the extracellular space, region, or matrix rather than from the nucleus or cytoplasm. Molecular functions (Fig. 11.4c) showed that the common functions were related to protein structure and disulfide bonds. These results were consistent with the information about secretion of proteases into surrounding tissues from the tumor during its growth, reproduction, and even invasion. Proteases from a tumor can cause hydrolysis of nearby proteins, which is beneficial for tumor growth and migration. The bioinformatics analysis of the 42 human-origin proteins is shown in Fig. 11.4.

11.9 Differential Host Nude Mouse Urinary Proteins

The criteria used to screen differential murine proteins were as follows: (1) Each protein contains at least two unique peptides. (2) The p-value of each protein is less than 0.05, and the fold change is greater than 2 or less than 0.5. (3) The protein was identified in all samples from the group (4/4). A total of 515 host-associated proteins were identified, and 8 proteins met these identification criteria. Table 11.2 provides detailed information.

The mouse differential protein information was compared with that from the Walker 256 tumor-bearing rat model and the glioma rat model (Table 11.3). This comparison revealed that the number of differential proteins in the PDX model was lower than the numbers in the other model tumor models under the same screening criteria.

Table 11.2 The eight differential urinary proteins in the nude mouse PDX model

Accession	Protein description	Group(s) discovered in	Unique peptides (No.)	Fold change	p-value	Trend	Homologous protein
Q5FW60	Major urinary protein 20 (Mup20)	Control group and tumor group	9	6.25	0.01051	Down	No
B0V388	Novel member of the major urinary protein (Mup) gene family	Control group and tumor group	5	5.88	0.02104	Down	No
A2ARV4	Low-density lipoprotein receptor-related protein 2 (Lrp2)	Control group and tumor group	77	2.73	0.04849	Up	Yes
P28843	Dipeptidyl peptidase 4 (Dpp4)	Control group and tumor group	17	2.97	0.03824	Up	Yes
Q03265	ATP synthase subunit alpha, mitochondrial (Atp5a1)	Tumor group	3	–	–	–	Yes
G3XA48	Isopentenyl-diphosphate Delta-isomerase 1 (Idi1)	Tumor group	3	–	–	–	Yes
G3UYJ7	Predicted gene 20,441 (Gm20441)	Tumor group	3	–	–	–	No
P52787	Gastric intrinsic factor (Gif)	Control group	5	–	–	–	Yes

Table 11.3 Differential urinary protein information from three tumor models

		PDX model	Glioma model	Walker 256 model
Experimental animal		Nude mouse	Rat	Rat
Immune system		T cell immunodeficient	Immunocompetent	Immunocompetent
Days		23	13	14
No. of differential proteins		8	27	31
No. of total proteins		515	778	533
Screening criteria	No. of unique peptides	> 2		
	Fold change	> 2 or < 0.5		
	p-value	< 0.05		
	Number in group	4/4	3/3	4/4

11.10 Differential Urinary Protein Functional Analysis of the Hosts

Biological process analyses were performed with 31 differential proteins from the Walker 256 tumor-bearing rat model and 8 differential proteins from the PDX model. The 8 differential proteins from the PDX model were related only to the transport process (the protein count was 3, and the *p*-value was 0.083).

However, 32 biological processes were found in the Walker 256 tumor-bearing rat model, many of which were related to immunity, including the complement activation classical pathway, acute phase response, defense response to bacterium, positive regulation of B cell activation, phagocytosis recognition, innate immune response, phagocytosis engulfment, inflammatory response, negative regulation of tumor necrosis factor production, B cell receptor signaling pathway, factor XII activation, apoptotic process, and immune system process (Table 11.4).

Table 11.4 Biological process analysis of the tumor-bearing rat and PDX nude mouse models

Model	Biological process(es)	Count	p-value
Walker 256 (includes only those biological processes with p-values less than 0.05)	Complement activation, classical pathway	5	8.40E-07*
	Acute phase response	4	5.90E-05
	Defense response to bacterium	5	1.40E-04
	Negative regulation of endopeptidase activity	5	2.10E-04
	Organ regeneration	4	7.80E-04
	Positive regulation of B cell activation	3	8.70E-04
	Phagocytosis, recognition	3	1.20E-03
	Innate immune response	5	1.70E-03
	Phagocytosis, engulfment	3	2.20E-03
	Inflammatory response	5	2.80E-03
	Negative regulation of tumor necrosis factor production	3	3.50E-03
	Response to drug	6	3.70E-03
	B cell receptor signaling pathway	3	3.80E-03
	Hemoglobin import	2	4.00E-03
	Vitamin metabolic process	2	4.00E-03
	Factor XII activation	2	6.00E-03
	Cobalamin transport	2	8.00E-03
	Proteolysis	5	1.40E-02

(continued)

Table 11.4 (continued)

Model	Biological process(es)	Count	p-value
	Positive regulation of dendritic cell chemotaxis	2	1.60E-02
	Response to lipopolysaccharide	4	1.80E-02
	Tissue remodeling	2	2.40E-02
	Aging	4	2.50E-02
	Carbohydrate metabolic process	3	2.60E-02
	Lipoprotein transport	2	2.80E-02
	Apoptotic process	4	3.60E-02
	Immune system process	2	4.90E-02
PDX (all biological processes)	Transport process	3	8.30E-02

*8.40E-07 = 0.00000084

In the urine from the tumor-bearing immunocompetent rat model, many differential proteins were associated with immune responses. In contrast to the rat model results, no host immune response proteins were found in the PDX model urine. The presence of these immune-related proteins explains why the number of urinary differential proteins in the PDX model was less than that of tumor rat models. Perhaps these immune-related proteins can be used as early urine candidate biomarkers of tumors. Due to the magnification effect of the immune system, even a small number of exogenous tumor cells can cause a strong reaction of the host system, with changes in the urine magnifying the changes caused by tumor secretion. No immune-related proteins were found in the urine of the PDX model because nude mice are immunodeficient. Therefore, immunodeficient animals may not be suitable models for searches of immunity-associated early tumor biomarkers in urine.

11.11 Conclusion

The 78 human-origin peptides successfully identified in the urine from the PDX nude mouse model at the peptide level originated from a colorectal tumor. In contrast to those of tumor-bearing immunocompetent rat models, host immune-related proteins in the PDX model were absent from the differential urinary proteins. Therefore, an immunodeficient animal, such as the nude mouse, may not be a suitable model for screening early immunity-associated colorectal tumor biomarkers in urine.

Acknowledgment Part of this chapter is based on published article: [1] Yongtao Liu, Youzhu Wang, Zhixiang Cao, Youhe Gao. Changes in the urinary proteome in a patient-derived xenograft (PDX) nude mouse model of colorectal tumor. Scientific report, 2019, 9(1): 4975.

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Chapter 12
Urine Is an Ideal Biomarker Resource in Early Detecting Neurodegenerative Diseases

Jing Wei

Abstract Neurodegenerative diseases include many different types. Alzheimer's disease (AD) is an incurable age-associated neurodegenerative disorder, which characterized by irreversible cognitive deficits and brain damage. Therefore, identification of candidate biomarkers before amyloid-β plaque deposition occurs is therefore of great importance for the early intervention of AD. Multiple sclerosis (MScl) is a chronic autoimmune demyelinating disease of the central nervous system and is difficult to diagnose in early stages. Without homeostatic mechanisms regulation, urine has the potential to accumulate changes in the whole body, which associated with AD and MScl earlier than cerebrospinal fluid and blood. This chapter highlights candidate urine biomarkers to detect AD before amyloid-β plaque deposition in the APP (swe)/PSEN1dE9 transgenic mouse model and to detect MScl when the clinical scores in the EAE group were 0 and no obvious histological changes were observed in an experimental autoimmune encephalomyelitis rat model. There were 29 urinary proteins changed in 4-month-old APP (swe)/PSEN1dE9 transgenic mice, which had not started to deposit amyloid-β plaque, 15 had been reported to be associated with AD, while 9 had been identified as AD biomarkers before. Thirty-one urinary proteins were altered in the 7-day experimental autoimmune encephalomyelitis rat model, and 17 of them were associated with neurological functions.

Keywords Alzheimer's disease (AD) · Multiple sclerosis (MScl) · Urine proteome · Early detection

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12.1 Introduction

Neurodegenerative diseases is the slow and progressive neuronal dysfunction characterized by the progressive loss of neurons in the central nervous system that leads to either functional loss (ataxia) or sensory dysfunction (dementia). Manifestation of neuronal loss or degeneration comes out in the form of memory impairment, locomotory dysfunction, cognitive defects, and emotional and behavioral problems. Neuronal degeneration is the main pathological feature of various age-related neurodegenerative diseases such as Alzheimer's disease (AD), Parkinson's disease (PD), multiple sclerosis (MS), Huntington's disease (HD), and amyotrophic lateral sclerosis (ALS) (Amor et al. 2014; Almeida-Santos et al. 2017).

Alzheimer's disease (AD) is a chronic age-associated neurodegenerative disorder associated with cognitive impairment and progressive dementia (Chen-Chen Tana and Tan 2014). As amyloid-β deposition can appear even in the stage 1 of preclinical AD patients, finding early clues in the early stages of AD, especially before amyloid-β plaque deposition, is urgent in today's AD research. Several studies have applied proteomic (Shevchenko et al. 2012) and metabolomic (Peng et al. 2014; Hu et al. 2012) analyses to characterize candidate biomarkers in plasma or brain tissues with mouse models, which were not sensitive enough for the early diagnosis of AD (before amyloid-β plaque deposition). Other studies have illustrated urine markers using samples from AD patients (Youn et al. 2011). However, all these studies were conducted after amyloid-β deposition had appeared in brain tissue and cognitive impairment or movement disorder symptoms had occurred. Therefore, there is an urgent need to find early candidate biomarkers before amyloid-β deposition.

Multiple sclerosis (MScl) is a chronic autoimmune demyelinating disease of the central nervous system (CNS), which is characterized by both inflammatory components and neurodegeneration (Compston and Coles 2002). The causes of MScl are not fully understood. Magnetic resonance imaging (MRI) is the most common diagnostic tool for MScl, and some clinical features may help to diagnose the disease. However, MRI lacks specificity for the early stages of the disease, and few clinical manifestations are specific to MScl. Some proteomic approaches have been used to investigate changes in urinary proteins/metabolites of MScl patients. For example, proteins such as trefoil factor 3 and lysosome-associated membrane protein 2 were differentially expressed in two phases (the third trimester of pregnancy and the postpartum period) of MScl patients, and these proteins may be associated with MScl (Singh et al. 2015). MScl shares many overlapping clinical features with neuromyelitis optica spectrum disorders (NMO-SD), but the treatment strategies differ substantially for these two diseases, and misdiagnosis can often result in wrong treatment (Trebst et al. 2014). Several urinary proteomic/metabolic studies have been conducted to differentiate these two diseases (Nielsen et al. 2015; Gebregiworgis et al. 2016). Therefore, it is necessary to diagnose MScl in the early phase clinically.

We think that without homeostasis, urine can accumulate many kinds of changes, and some of these changes are associated with disease and will become biomarkers (Gao 2013, 2014). Using animal models can reduce the influence of genetic and environmental factors on the urine proteome, which will establish the direct association with disease.

12.2 Urinary Proteomic Discovery

12.2.1 Alzheimer's Disease (AD)

To searching early candidate urinary biomarkers before amyloid-β deposition, the amyloid precursor protein (APP) (swe)/PSEN1^{dE9} transgenic mice, overexpressing mutant APP and PS1 (APP/PS1), have been widely used as a model of AD to elucidate the pathogenic processes of the disease and to investigate candidate biomarkers (Sun et al. 2015). Deposition of amyloid-β in the hippocampus of 6-month-old mice occasionally occurred (Laursen et al. 2013; Kilgore et al. 2010), and a mass of amyloid-β had been deposited in the hippocampus of 8-month-old mice (Holcomb et al. 1998; Garcia-Alloza et al. 2006). However, the cognitive abnormality of APP (swe)/PSEN1^{dE9} transgenic mice appeared at 4 months, when amyloid-β plaques had not yet deposited (Bonardi et al. 2011). Potential candidate urinary biomarkers that appear before brain pathology can thus be identified by using 4-month-old APP (swe)/PSEN1^{dE9} transgenic mice (Figure 12.1).

Urine samples from 4-month-, 6-month-, and 8-month-old mice in transgenic (n = 3, each month) and wide-type control groups (n = 3, each month) were col-

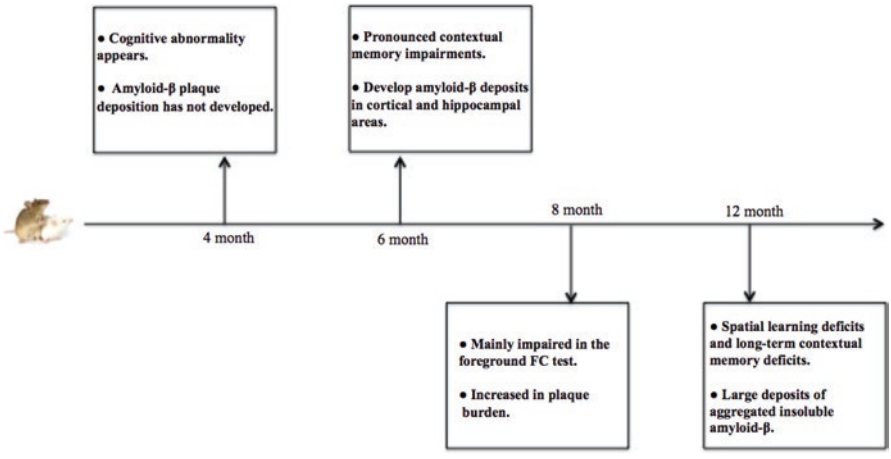

Fig. 12.1 Characteristics of behavioral and pathological profiles of different stages of APP (swe)/PSEN1^{dE9} transgenic mice

lected in metabolic cages. During urine collection, all rats were given free access to water without food to avoid contamination. Then, urine was centrifuged, precipitation by three volumes of acetone, and resuspended in lysis buffer (8 M urea, 2 M thiourea, 25 mM dithiothreitol (DTT), and 50 mM Tris). Protein concentrations were measured using the Bradford method. All urinary proteins were digested with trypsin using in-gel protein digestion (Speicher et al. 2000) or filter-aided sample preparation methods (Wisniewski et al. 2009). Digested peptides were analyzed by using label-free and TMT-labeling proteomic analysis.

12.2.2 Multiple Sclerosis (MScl)

Because MScl is difficult to diagnose at early stages in clinical practice, the urinary proteome of an experimental autoimmune encephalomyelitis (EAE) model was used for the discovery of early biomarkers of MScl. EAE is the most commonly employed model for MScl (Robinson et al. 2014), and it has been a powerful tool for studying relevant mechanisms in MScl as well as for translating the findings into clinically meaningful therapeutic approaches (Ben-Nun et al. 2014). The clinicopathologic characteristics of the EAE model, including inflammation and demyelination of the CNS, are similar to those of MScl (Merkler et al. 2006). Thus, studies using the EAE model have provided new insights into the pathogenesis and pathophysiology of MScl.

EAE was induced in Lewis rats with myelin basic protein (MBP) as previously reported (Mannie et al. 1985). Rats were placed in metabolic cages on days 0 (before MBP immunization, baseline), 7, 14, and 21 to collect urine. Each time three pairs of rats in the two groups were sacrificed, and tissues were collected for histological analyses. Body weight and neurological impairment scores were evaluated daily. The progression of EAE was measured daily based on neurological impairment and scored from 0 to 5 as follows (Schneider et al. 2009): grade 0, no symptoms; 0.5, mild floppy tail; 1, floppy tail; 2, hind limb weakness; 3, severe paraparesis; 4, tetraparesis; and 5, moribund. On day 7 after immunization, the rats with EAE began to display some clinical symptoms, and this phenomenon was identified as the time point to study early candidate biomarkers. Six urine samples (from three rats with EAE and three control rats) collected on day 7 were used for MS analysis. Peptides in each sample were labeled with 126, 127, 128, 129, 130, and 131 tandem mass tag (TMT) reagents (Thermo Fisher Scientific) according to the manufacturer's instructions. The labeled peptides were mixed and then analyzed with two-dimensional liquid chromatography-MS/MS (LC-MS/MS).

12.3 Identified Differential Proteins

12.3.1 Alzheimer's Disease (AD)

Urine samples of APP (swe)/PSEN1^{dE9} transgenic mice at three growth stages (4-, 6-, and 8-month-old) were collected and analyzed by using label-free and TMT-labeling proteomic analysis. There were 29, 33, and 86 urinary proteins changed significantly on 4 -, 6-, and 8-month-old transgenic mice, respectively, with the following criteria: (i) compared with the control group, fold change ≥ 1.5 or ≤ 0.67 and (ii) p-value <0.05.

Among these 29 significantly changed proteins on 4-month-old transgenic mice, 13 had been reported to be associated with the pathology mechanisms of AD, while 9 were identified as direct AD biomarkers (Table 12.1). Seven proteins relate to the development of AD as well as biomarkers of AD. (1) Kallikrein-1 (KLK1) levels were different in 4-, 6-, and 8-month-old groups than the wild-type group, and the kallikrein-kinin system mediates inflammation in AD in vivo (Buck. TAVaHS 2011). Kallikrein-6, one member of the kallikrein family, was reported as a biomarker of AD and to have increased levels in CSF and serum and decreased levels in tissue (Diamandis et al. 2000). (2) Galactocerebrosidase (GALC) levels were different in 4- and 8-month-old groups than the wild-type group, and a deficiency in galactosylceramidase potentially contributed to neurodegenerative disease (Marshall and Bongarzone 2016). Ceramide has been suggested to participate in the neuronal cell death that leads to AD. GALC, as a gene connected to ceramide metabolism, was upregulated in the brain tissue of AD patients, making it an attractive candidate for diagnostic purposes and for intervening in neurodegenerative processes (Filippov et al. 2012). (3) Ceruloplasmin (CERU) levels were higher in both 4- and 8-month-old groups than in the control group, with the same trend of increased levels found in the serum of AD patients (Park et al. 2014). The levels of CSF diagnostic markers, such as Aβ42, tau, and phospho-tau, were correlated with lower plasma copper and CERU levels in patients with Alzheimer's disease (Kessler et al. 2006). The ratio of CERU concentrations measured by enzymatic methods (eCP) to those measured by immunological methods (iCP), eCP/iCP, reflects the high specificity of AD patients as well as a decreased risk of having AD (S et al. 2016). CERU had less ferroxidase activity in AD patients than wild-type patients, which contributed to the development of AD (Bush 2013). (4) Fibronectin (FINC) levels were different in 4- and 8-month-old groups than in the wild-type group and were reported to be a novel biomarker for AD from blood (Long et al. 2016). In addition, FINC levels were significantly lower in plasma in mild cognitive impairment (MCI) patients than healthy patients, which provides further insight into the biological pathways and processes that underpin the pathophysiology and progression of MCI and AD

Table 12.1 Details of differential urinary proteins of (APP) (swe)/PSEN1^{dE9} transgenic mice on 4-month-old

MW	Protein name	UniProt	Human UniProt	P-value	Fold change	Confidence score	Normalized abundance						Pathology and mechanism	Biomarkers
							WT-1	WT-2	WT-3	AD-1	AD-2	AD-3		
>85 kDa	Cadherin-1	P09803	P12830	0.022	1.87	218.7	2,619,939	3,821,894	4,545,058	7,387,696	5,702,665	7,448,193	Zhenwei Shang et al. (2015); Seong et al. (2015)	–
	Keratin, type II cytoskeletal 2 oral	Q3UV17	Q01546	0.041	0.56	181.9	95,619	152,124	160,984	52,841	108,167	67,233	–	–
50–85 kDa	Ceruloplasmin	Q61147	P00450	0.018	3.19	142.8	1,405,128	700,799	1,034,296	4,233,275	2,979,048	2,817,111	S et al. (2016); Bush (2013)	Kessler et al. (2006)
	Alpha-amylase1	P00687	P04745	0.037	2.98	1271.6	16,862,562	16,394,995	16,747,254	27,733,747	36,333,676	85,064,392	John et al. (1995)	–
	Ectonucleotide pyrophosphatase	Q9R1E6	Q13822	0.038	2.89	438.4	6,410,477	4,227,746	3,261,439	14,447,745	15,522,911	10,137,661	–	Heywood et al. (2015)
	Annexin A11	P97384	P50995	0.035	2.54	124.5	112,847	385,493	224,516	851,694	561,465	420,834	McArthur et al. (2010)	Yamaguchi et al. (2010)
	Angiotensinogen	P11859	P01019	0.036	2.53	340.3	1,832,470	6,008,159	4,047,815	8,130,284	9,777,242	12,155,633	Savaskan (2005)	Mateos et al. (2011)
	Kallikrein-1	P15947	P06870	0.028	2.55	161.4	13,993,666	15,481,983	8,200,572	32,915,114	24,952,991	38,216,076	Buck. TAVaHS (2011)	Diamandis et al. (2000)
	Pantetheinase	Q9Z0K8	O95497	0.010	2.25	329.7	7,264,591	9,291,049	8,941,437	18,429,885	12,901,960	26,056,688	–	–
	Sulfhydryloxidase1	Q8BND5	O00391	0.029	1.93	444.1	4,216,654	7,066,885	5,327,850	12,135,722	10,500,098	9,505,950	–	–
	Sphingomyelin phosphodiesterase	Q04519	P17405	0.004	1.71	138.9	789,408	934,631	809,095	1,433,994	1,281,043	1,625,436	Filippov et al. (2012); Alessenko and AEBaLBD (2004); He et al. (2010)	–
	N-acetylmuramoyl-L-alanine amidase	Q8VCS0	Q96PD5	0.001	1.63	237.3	4,431,595	4,089,554	4,317,879	6,991,718	6,218,791	7,761,766	–	–

	Galactocerebrosidase	P54818	P54803	0.002	1.51	296.1	23,253,378	21,116,228	25,380,610	39,727,782	31,492,109	33,948,684	Marshall and Bongarzone (2016)	Filippov et al. (2012)
30–50 kDa	Carbonic anhydrase 2	P00920	P00918	0.050	7.64	139.9	1,026,487	137,540	1,196,867	2,483,019	8,183,767	7,363,671	Jang et al. (2010)	–
	ATP-binding cassette sub-family A member 13	Q5SSE9	Q86UQ4	0.003	3.97	186.0	162,885	413,064	239,944	1,326,933	1,045,477	870,531	–	–
	Protein LEG1 homolog	Q8C6C9	Q6P5S2	0.046	3.17	331.5	617,434	5,666,756	2,910,064	10,253,781	9,689,764	9,179,997	–	–
	Cathepsin B	P10605	P07858	0.029	3.13	338.5	7,139,961	1,114,522	1,317,986	9,277,618	8,644,600	12,074,430	Hook et al. (2014)	Sun et al. (2015)
	Acid ceramidase	Q9WV54	Q13510	0.031	0.42	264.6	39,183,681	22,168,743	30,757,070	11,403,664	10,956,829	16,671,159	Huang et al. (2004)	–
	Sulfhydryl oxidase 1	Q8BND5	O00391	0.005	0.40	271.4	6,515,045	6,236,645	3,506,111	2,174,726	1,897,719	2,494,845	–	–
	Eosinophil cationic protein 1	P97426	No	0.031	0.39	167.5	251,235,145	103,629,026	273,458,396	112,170,010	66,736,059	65,435,575	–	–
	Fibronectin	P11276	P02751	0.017	0.36	191.7	6,815,535	4,157,474	3,089,723	1,594,554	2,037,884	1,485,064	Muenchhoff et al. (2015)	Long et al. (2016)
	Prostaglandin-H2 D-isomerase	O09114	P41222	0.018	0.27	196.1	116,338,435	118,916,422	35,968,640	25,071,521	28,557,837	19,099,588	–	–
	Dipeptidyl peptidase 2	Q9ET22	Q9UHL4	0.017	0.23	329.3	36,130,176	10,831,853	12,769,425	4,129,725	4,626,310	4,946,023	–	–
	Lipoprotein lipase	P11152	P06858	0.025	0.18	157.9	12,700,373	4,762,033	4,358,911	1,006,139	706,457	2,137,022	Gong et al. (2013)	–
	Sialate O-acetylesterase	P70665	Q9HAT2	0.041	0.17	150.5	5,186,756	1,470,459	629,510	242,721	323,999	669,184	–	–
	Alpha-1-antitrypsin 1–4	Q00897	No	0.029	0.09	386.0	47,453,084	7,866,154	10,014,171	1,352,876	1,413,734	3,276,721	–	–

(continued)

Table 12.1 (continued)

MW	Protein name	UniProt	Human UniProt	P-value	Fold change	Confidence score	Normalized abundance						Pathology and mechanism	Biomarkers
							WT-1	WT-2	WT-3	AD-1	AD-2	AD-3		
15–30 kDa	Deoxyribonuclease-1	P49183	P24855	0.002	2.46	177.0	6,661,598	5,715,618	7,105,781	11,646,345	22,351,861	13,973,828	–	–
	Ig kappa chain C region	P01837	P01834	0.014	1.80	103.9	8,822,671	11,713,287	12,329,617	21,590,819	21,713,190	15,916,672	–	Shen et al. (2016)
<15 kDa	Deoxyribonuclease-1	P49183	P24855	0.026	2.47	146.4	1,360,757	728,821	1,080,584	1,869,443	3,272,391	2,687,073	–	–
	Secretoglobin family 2B member 20	Q9JI02	No	0.026	0.56	323.6	15,981,747	29,749,962	27,032,579	15,560,357	9,828,085	15,548,114	–	–
	Ig kappa chain V-V region L7 (fragment)	P01642	No	0.021	0.20	130.7	893,023	1,868,961	641,920	208,754	175,548	309,125	–	–

(Muenchhoff et al. 2015). (5) Cathepsin B (CATB) levels were different in 4- and 8-month-old groups than the wild-type group. CATB has been previously reported to be upregulated in brain tissues from APP/PS1 transgenic mice, and its levels changed in the same direction relative to healthy mice as they changed in the serum of AD patients relative to control patients, making CATB a potential biomarker of AD (Sun et al. 2015). In addition, CATB produces brain pyroglutamate amyloid-β, which represents a potential AD therapeutic (Hook et al. 2014). (6) Angiotensinogen (ANGT) was upregulated in 4-month-old groups and has exhibited increased levels in the CSF of AD patients (Mateos et al. 2011). As a component of the renin-ANGT system (RAS), ANGT is helpful for AD processes (Savaskan 2005). (7) Annexin A11 (ANX11) was upregulated in 4-month-old groups. Annexin A1 was reported to be expressed strongly in the microglia of AD patients, which aids the surveillance of microglia and the maintenance of brain homeostasis by using Annexin A1-dependent mechanisms (McArthur et al. 2010), and Annexin A5 was reported to be a biomarker of AD and present at increased levels in the plasma of AD patients (Yamaguchi et al. 2010). The protein-normalized abundances and spectral counts of all seven differential proteins were greater in every mouse in the high-abundance group than those in the low-abundance group. Two other differential proteins have been mentioned as potential AD biomarkers. (1) Ectonucleotide pyrophosphatase/phosphodiesterase family member 2 (ENPP2) levels were higher in the CSF of AD patients than the CSF of healthy controls. ENPP2 can be used to specifically discriminate AD from Lewy body dementia, making it a candidate AD biomarker (Heywood et al. 2015). (2) The Ig kappa chain C (IGKC) region was reported to be a potential serum biomarker of AD at the early stage (Shen et al. 2016).

12.3.2 Multiple Sclerosis (MScl)

As indicated above, on day 7, the clinical scores in the EAE group were “0” and were similar to the scores in the control group; no obvious histological changes were observed. Therefore, urine samples collected on day 7 after immunization were used for early biomarker detection. Statistical analyses indicated that 31 proteins were significantly affected by MBP immunization (15 up- and 16 downregulated proteins). Some of them had been reported to be associated with MScl. For example, (i) elevated levels of kininogen and C9 in the CSF have been reported in rats that have EAE. Both kininogen (a precursor for kinin) and complement component C9 are mediators of inflammation and play important roles in response to inflammatory injury (Rosenling et al. 2012). (ii) Protease family members, including metalloproteases, serine proteases, and cysteine proteases, can be markers of disease activity in MScl. The neutrophil collagenase MMP-8, a metalloprotease, has been shown to increase in the CNS in response to EAE and is correlated with symptom severity (Scarisbrick 2008). (iii) ANGT is involved in maintaining blood pressure and in the pathogenesis of essential hypertension and preeclampsia.

Interestingly, the upregulation of serum angiotensin-converting enzymes is related to disease activity in longitudinal analysis (Constantinescu et al. 1997). (iv) Serum fatty acid binding protein (FABP) is thought to distinguish subtypes of MScl, because it is expressed at the highest level in secondary progressive MScl and increased during early stages of pediatric-onset MScl (Messina et al. 2013). (v) Oncomodulin, a factor produced by macrophages, promotes axon growth in neurons and is an indicator of CNS injury (Flanders et al. 1998), including MScl. In the early stages of EAE, the levels of oncomodulin are reduced, which may partly be due to axonal injury. Other differential proteins were listed in the preprint (Zhao et al. 2017).

12.4 Functional Enrichment Analysis

12.4.1 Alzheimer's Disease (AD)

Functional annotation of differential urinary proteins was performed using DAVID (Liu and Cao 2016). The differential proteins at three time points were classified to be involved with certain biological processes (Figure 12.2), molecular components, and molecular functions. In 4-month-old mice, differential urine proteins involved in lipid metabolism and lipid catabolism were enriched. As lipid metabolism is a fundamental process for brain development and function, aberrant lipid metabolism was not surprisingly common in an animal model of AD that relates to AD pathogenesis (Hamilton et al. 2015; Liu and Zhang 2014; Giannopoulos et al. 2014). For example, AD-induced perturbation of niche fatty acid metabolism can suppress the homeostatic and regenerative functions of neural stem cells, supporting the mechanism of AD pathogenesis (Hamilton et al. 2015). Abnormal lipid metabolism influences Aβ metabolism and deposition in both brain parenchyma and vasculature, as well as tau hyperphosphorylation and aggregation, which then likely triggers a

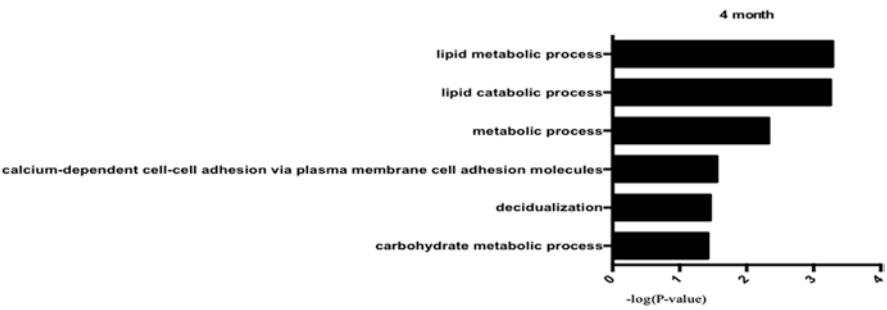

Fig. 12.2 Biological processes of differential proteins on 4-month-old APP (swe)/PSEN1^{dE9} transgenic mice

series of downstream catalytic events that eventually affect the progression of AD pathogenesis (Liu and Zhang 2014). More importantly, lipid rafts from human cerebral cortex are associated with the pathogenesis of early AD, as β-secretase/AβPP (amyloid-β protein precursor) interactions and lipid raft microviscosity are strongly and positively correlated in AD frontal and entorhinal cortices, indicating that the aberrant lipid metabolism had already occurred in the early stage of AD (Mario Díaz et al. 2015). Decidualization and calcium-dependent cell-cell adhesion via plasma membrane cell adhesion molecules, which are not reported in AD studies, may play roles in the mechanism of AD pathology. Recent studies demonstrated that using a triple receptor agonist (TA), which activates GIP-1, GIP, and glucagon receptors at the same time, reduces the total amount of amyloid-β in an APP/PS1 transgenic mouse model through a 2-month TA treatment (Tai et al. 2017). However, this research used 6-month-old transgenic mice for a 2-month treatment, and amyloid-β had already appeared. We thus suppose that a more effective treatment can be achieved when the 4-month-old transgenic mice are used for therapy. The top-ranked process of 4-month-old APP (swe)/PSEN1^{dE9} transgenic mice was lipid metabolic process, and the galactocerebrosidase, acid ceramidase, ectonucleotide pyrophosphatase, prostaglandin-H2 D-isomerase, and lipoprotein lipase were included in this process.

12.4.2 Multiple Sclerosis (MScl)

The altered 31 deregulated proteins were uploaded into IPA and determined their interactions with network proteins associated with "neurological diseases" (Figure 12.3). A total of 17 deregulated molecules were identified in this functional interaction. In particular, specific peptidases (C4, CPB2, CTSB, MMP-7, and MMP-8) and enzymes (CP, PPIA, and TXN) were identified as being altered in MBP-induced EAE.

The hub proteins identified in the neurological disease networks are IL-1, LDL, and P36 MAPK. The upregulation of P38 MAPK is considered to be closely related to 4-1BB signaling in T cells and is consistent with the induction of EAE because EAE is initiated by immunization with autoantigens presented to MHC class II-restricted CD4+ T helper (Th) cells (Robinson et al. 2014). Additionally, the inhibition of active mouse p38 MAPK in CD4+ T cells was shown to decrease the severity of EAE in mice (Noubade et al. 2011). IL-1 is one of the commonly used inflammatory factors. Although it is rarely detected in the normal brain, IL-1 is significantly upregulated and plays a central role in neuroinflammation, especially under neurodegenerative conditions (Spulber et al. 2009). Therefore, the proteins deregulated in response to MBP immunization include interactors that preferentially function in CD4+ T cells, neuroinflammation, and lipid metabolism and hence may affect neurological functions.

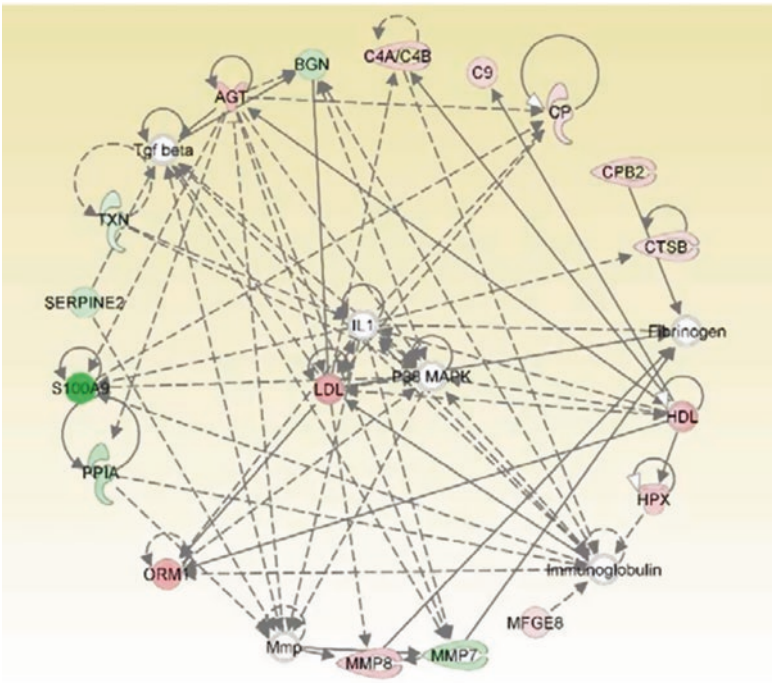

Fig. 12.3 Network related to neurological functions annotated by IPA. Red indicates upregulated genes, and green indicates downregulated genes. Blank indicates that the gene was not deregulated in the study

12.5 Conclusions and Future Prospects

Our results indicated that (i) urine proteins enable AD early detection before amyloid-β plaque deposition, which may provide an opportunity for intervention, and (ii) urine proteome may provide clues for the pathogenesis of MScl, indicating urine can be a good source of MScl early biomarkers. In future studies, clinical urine samples of AD and MScl patients will be used to validate the differential proteins identified in this study. We think that more neurodegenerative diseases will be deserved to find early biomarkers using animal models through urine proteomics.

Acknowledgments Part of this chapter is based on published articles: Fanshuang Zhang∗, Jing Wei∗, Xundou Li∗, Chao Ma#, and Youhe Gao#. Early Candidate Urine Biomarkers for Detecting Alzheimer's Disease Before Amyloid-beta Plaque Deposition in an APP (swe)/PSEN1dE9 Transgenic Mouse Model [J]. *Journal of Alzheimer's disease*, 2018, 66(2): 613–637.

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Chapter 13
Urinary Proteome Biomarkers for Early Detection of Respiratory Diseases

Jianqiang Wu and He Huang

Abstract Idiopathic pulmonary fibrosis (IPF) is a progressive and devastating lung disease with a very poor prognosis. Chronic obstructive pulmonary disease (COPD) is a chronic inflammatory airway disease and the third leading cause of death worldwide. Disease biomarkers are highly desired for IPF and COPD, especially in early disease phase. Urine is an ideal biomarker source and has the potential to reflect small and early pathological changes. This chapter introduces the application of urine proteomics in biomarker discovery of these two diseases. A bleomycin-induced model and a smoking-induced model were used to mimic the pathophysiological process. Using proteome quantitation, the results showed that urine proteins changed significantly before obvious histopathological changes in lungs. Moreover, early detection and prompt treatment could effectively inhibit pulmonary fibrosis, whereas the same treatment at a late disease phase had very limited therapeutic effects. Our findings will improve the understanding of the pathogenesis of IPF and COPD and accelerate urine biomarker discovery in respiratory diseases.

Keywords Idiopathic pulmonary fibrosis · Chronic obstructive pulmonary disease · Urine · Proteomics · Biomarkers · Early detection · Monitoring · Treatment

13.1 Introduction

Idiopathic pulmonary fibrosis (IPF) is a progressive and lethal lung disease. As few effective drugs are available (King Jr. et al. 2014), IPF has a very poor prognosis with an average survival time of 3–5 years, leading to an increasing morbidity and mortality (Raghu et al. 2014; Navaratnam et al. 2011). Chronic obstructive pulmonary disease (COPD) is characterized by a progressive and irreversible airflow obstruction, and it is one of the leading global causes of morbidity and mortality

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(Moon et al. 2018). Currently, many challenges remain in early detection of IPF and COPD (Maher 2013; Cagnone et al. 2018), and hardly any drug can reverse their progression in late disease phases. Therefore, early disease biomarkers are highly desired for IPF and COPD.

Proteomics is a powerful tool for biomarker discovery. The samples for biomarker discovery using proteomics approaches in pulmonary diseases include lung tissues, bronchoalveolar lavage fluid (BALF), sputum, blood, and urine. Among them, urine can be collected in a noninvasive manner with large volumes. Moreover, without homeostatic control urine can accumulate changes in the body; thus urine has the potential to reflect early and small pathological changes (Gao 2013; Wu and Gao 2015). Despite these advantages, urine proteomics has been underutilized in pulmonary diseases. Some studies have applied proteomics to identify potential biomarkers of IPF and COPD (Foster et al. 2015; Niu et al. 2017; Korfei et al. 2013; Baralla et al. 2018; Lopez-Sanchez et al. 2017), whereas urinary biomarker studies are very limited (Iguchi et al. 1998; Carleo et al. 2017). In anatomical localization, urine is far from the lung, and it is unsure whether pulmonary pathological changes can be detected in the urine. Meanwhile, whether differential proteins identified in tissue or other bio-fluids can also be identified with differential abundance in the urine as noninvasive biomarkers remains unclear.

Bleomycin (BLM) can induce damage to alveolar epithelial cells and activate fibroblasts and subsequent lung fibrosis. Cigarette smoking is the primary risk agent for COPD. In this chapter, a BLM-induced rat model and a cigarette smoking-induced rat model were used to mimic the pathophysiological processes of IPF and COPD. We performed labeled proteomics analyses to identify candidate urine biomarkers in early fibrosis phase, during IPF progression and after drug treatment. We also performed label-free quantification to analyze changes of urine proteomes at three time points during the progression of smoking-induced COPD.

13.2 Disease Model Establishment

After male SD rats (200–220 g) were anesthetized, bleomycin solution (4 mg/ml) was dripped into the trachea (3 mg/kg body weight) to induce lung fibrosis. Dynamic histopathological changes of lungs were showed in Fig. 13.1. Based on Masson's trichrome staining, it was observed that only a very slight fibrosis appeared in the lungs on day 7, a limited interstitial fibrosis existed on day 14, and quite a lot of collagen fibers existed on day 21. These results suggested that typical lung fibrosis appeared on day 21 after BLM induction.

To establish a COPD model, commercial non-filtered cigarettes (trade name: DA QIAN MEN) containing 11 mg of tar and 0.8 mg of nicotine per cigarette were used. Male Wistar rats (weighting 180–200 g) were placed in a chamber with size of 36 cm (length) × 20 cm (width) × 28 cm (height). The smoke of a cigarette was delivered into the chamber and six cigarettes for 1 h in the morning and six cigarettes 1 h in the afternoon for 6 days a week. The lungs of the rats were removed at week

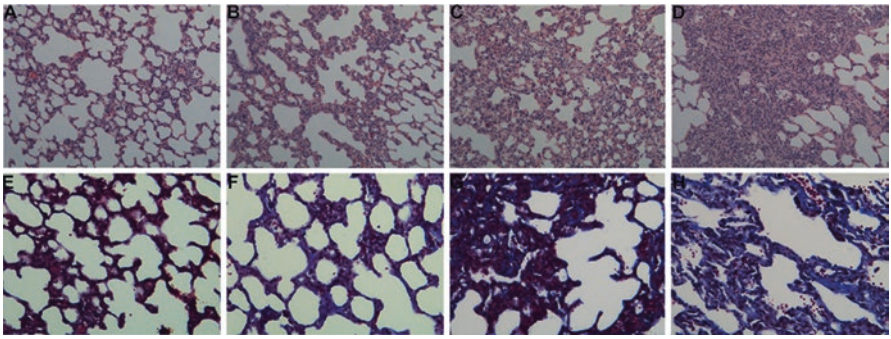

Fig. 13.1 Pathological changes in the lungs of BLM-induced rats. H&E staining (**a**–**d**) and Masson's trichrome staining (**e**–**h**) of lungs on days 7, 14, 21, and 28 after instillation. (This figure is from Wu et al. 2017)

2, week 4, and week 8 and fixed in 10% neutral-buffered formalin for histopathological analysis. At week 2, there was no significant change in the bronchus; at week 4, bronchial epithelial detachment was observed; at week 8, the rat lung bronchial epithelial cells were denatured, adhered, and partially detached (Fig. 13.2). In addition, at week 2, there were no significant changes in alveoli and bronchial epithelial goblet cells using HE and AB-PAS staining.

13.3 Changes in the Urinary Proteome of Rats with Lung Fibrosis

Urine samples from three random BLM rats on days 0 and 6 after induction were used to identify early detection biomarkers. Six protein samples were digested with trypsin, labeled with 6-plex TMT reagents, and analyzed using 2D LC-MS/MS. A total of 522 urine proteins were identified, and 10 differential proteins were screened ($\geq$2 unique peptides, $p < 0.05$, and fold change $\geq$1.5) (Table 13.1). We found that several differential urine proteins were associated with lung fibrosis. Vimentin is a tissue fibrosis marker, and it also increased in the urine of rats with kidney fibrosis (Yuan et al. 2015). Low-density lipoprotein receptor-related protein 4 may negatively regulate the Wnt pathway, which is involved in tissue fibrosis and is associated with IPF (Chen et al. 2016). Collagen alpha-1(I) chain significantly upregulated in the urine of model rats, and abnormal deposition of extracellular matrix is a necessary process in IPF. Thus, these urinary proteins were potential protein biomarkers for early detection of IPF.

To monitor disease progression, urine samples from BLM-induced rats were collected on days 0, 9, 14, and 21. Urine proteins of two model rats at four time points were prepared and labeled with 8-plex iTRAQ reagents. Using 2D LC-MS/MS and iTRAQ quantification, a total of 684 urine proteins and 30 differential proteins were identified. The dynamic changes of these differential proteins at different time

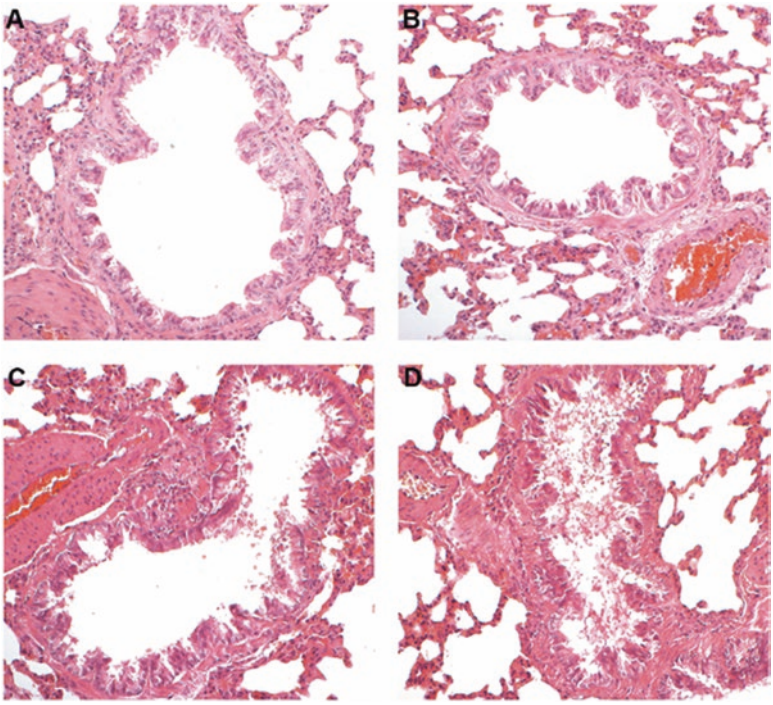

Fig. 13.2 Histological changes of the bronchial tissues after inhalation of cigarette smoke (CS). (**a**) Control group, (**b**) CS exposure for 2 weeks, (**c**) CS exposure for 4 weeks, and (**d**) CS exposure for 8 weeks H&E magnification: 200×. (This figure is cited from Huang et al. 2018)

Table 13.1 Differential urinary proteins at early stage of pulmonary fibrosis

		Change ratio to day 0			*P*
Accession	Protein name	Rat 1	Rat 2	Rat 3	
P02761	Major urinary protein	2.76	1.69	3.00	<0.001
P14046	Alpha-1-inhibitor 3	0.65	0.65	0.65	<0.001
P02454	Collagen alpha-1(I) chain	0.31	0.63	0.44	0.010
P31000	Vimentin	2.83	1.69	1.65	0.014
Q10758	Keratin, type II cytoskeletal 8	1.50	1.95	3.95	<0.001
Q63751	Vomeromodulin (fragment)	1.91	3.27	1.75	0.001
Q5M7T9	Threonine synthase-like 2	1.61	3.24	2.37	0.002
Q9QYP1	Low-density lipoprotein receptor-related protein 4	3.30	2.38	2.31	0.011
P48721	Stress-70 protein, mitochondrial	1.55	2.04	1.72	0.023
Q00981	Ubiquitin carboxyl-terminal hydrolase isozyme L1	1.60	2.14	1.96	0.024

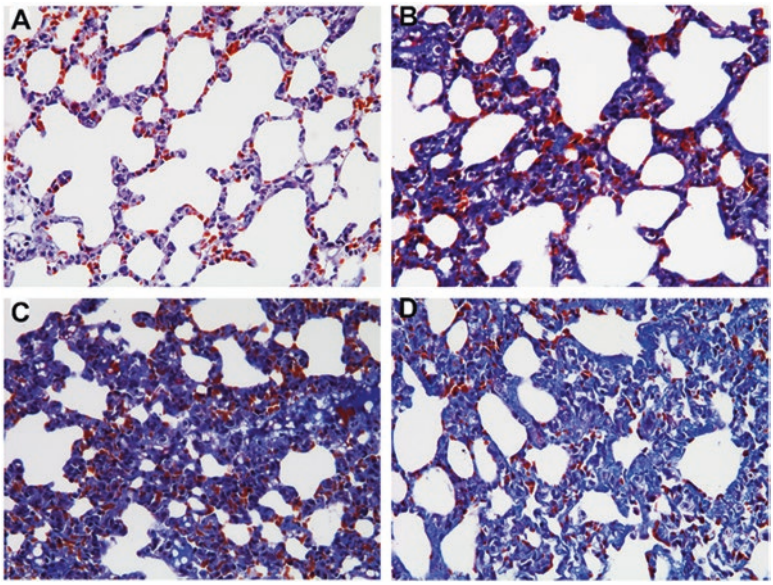

Fig. 13.3 Histopathological results of lungs of BLM-induced rats after treatment at different time points. (**a**–**c**) Prednisone treatment beginning on days 8, 15, and 22 after BLM induction. (**d**) BLM group treated with saline. The endpoint was 28 days after BLM induction. The magnification of Masson's trichrome staining was 100×. (This figure is cited from Wu et al. 2017)

points suggested urine proteins have the potential as candidate biomarkers to monitor disease progression. These 30 differential proteins were uploaded in IPA software for pathway analysis. The top canonical pathways included acute phase response pathway, the intrinsic prothrombin activation pathway, and IGF-1 pathway. In previous studies, a prothrombotic state was reported to be associated with more severe disease and earlier mortality in IPF (Navaratnam et al. 2014; Fahim et al. 2014). IGF-1 pathway is also associated with IPF, and blockading this pathway can hasten disease resolution in BLM-induced lung injury (Choi et al. 2009). In addition, four differential proteins identified during disease progression were further validated by Western blot, including insulin-like growth factor-binding protein 3 (IGFBP3), WNT1-inducible signaling pathway protein 1 (WISP1), calbindin, and fibulin-5. In previous studies, IGFBP3 and fibulin-5 were differentially expressed in tissue of lung fibrosis (Decaris et al. 2014), and WISP1 was a candidate therapeutic target for the attenuation of lung fibrosis (Brissett et al. 2012). The overall decreased trend of these four proteins in Western blot was consistent with their MS results.

Then, prednisone treatment (4 mg/kg/day) was performed on days 8, 15, and 21 after BLM instillation to the intervention groups. The control group was administered with equal saline. Treatment response was evaluated on day 28 using the lung histopathology. As shown in Fig. 13.3, the results revealed that early prednisone treatment effectively inhibited lung fibrosis, whereas treatment at a later phase had very limited effects, suggesting early detection of urine biomarker may lead to early

Table 13.2 The change trends of five urine proteins were reversed after treatment

Accession	Protein description	Fold changes	
		Lung fibrosis	After treatment
P02625	Parvalbumin alpha	↓ 1.81	↑ 1.67
Q9WVH8	Fibulin-5	↓ 1.54	↑ 1.50
Q6RY07	Acidic mammalian chitinase	↑ 2.55	↓ 2.24
P07171	Calbindin	↓ 2.50	↑ 1.75
P12020	Cysteine-rich secretory protein 1	↑ 2.70	↓ 1.61

effective treatment for better prognosis in IPF. In addition, urine samples from another model group (21 days after BLM induction) and another treatment group (treatment for 7 days after BLM induction for 14 days) were used to identify therapeutic biomarkers using 4-plex TMT quantification. As a result, five urine proteins exhibited opposite change trends during disease progression and after prednisone treatment (Table 13.2), suggesting they are candidate therapeutic biomarkers for IPF.

13.4 Differential Urinary Proteins in the COPD Rat Model

Urine samples of the COPD group and control group were collected at week 2, week 4, and week 8. By LC-MS/MS, a total of 547 urine proteins were identified, and 21 differential proteins were screened (≥2 unique peptides, $p < 0.05$, and fold change ≥1.5). There were eight significantly differentially expressed proteins at week 2, seven significantly changed proteins at week 4, and five significantly changed proteins at week 8 (Table 13.3). After smoking for 2 weeks when there were no significant pathological changes, eight differential proteins were identified: two proteins had been reported to be biomarkers of COPD, while four proteins were associated with COPD. For example, vitamin D-binding protein was significantly downregulated in smoker plasma and could be used as a candidate marker of smoking-induced disease (Bortner Jr. et al. 2011), and its levels in serum inversely correlate with lung function in COPD (Wood et al. 2011). Fetuin-A is a clinically relevant biomarker in patients with COPD that may be useful in the identification of exacerbation-prone patients (Minas et al. 2013). As for trefoil factor-1 (TFF-1), the secretion of trefoil factor peptides was increased in the sputum, serum, and BALF samples from COPD patients, and TFF-1 expression was significantly upregulated compared with the controls (Viby et al. 2015a; Viby et al. 2015b).

13.5 Clinical Prospects

Interestingly, some differential proteins identified in our BLM-induced model are associated with IPF pathogenesis. For example, the transcriptional activation of angiotensinogen is a critical step in IPF (Dang et al. 2013). IGFBP-3 is involved in

Table 13.3 Differentially expressed proteins in the urine of COPD rats

Accession	Protein description	Trend	Time points	Fold change	COPD correlation	COPD biomarker
P02651	Apolipoprotein A-IV	↑	2 W	4.04		
P04276	Vitamin D-binding protein	↑	2 W	2.54	Chen et al. (2015)	Wood et al. (2011)
Q01177	Plasminogen	↑	2/4 W	2.37/2.27	Waschki et al. (2017)	
P24090	Fetuin-A	↑	2/4 W	2.22/2.94	Alpsoy et al. (2014)	Minas et al. (2013)
Q05175	Brain acid soluble protein-1	↑	2 W	1.99		
Q63467	Trefoil factor-1	↑	2 W	1.86		Viby et al. (2015a, b)
Q5XI43	Matrix remodeling-associated protein-8	↑	2 W	1.63		
O70513	Galectin-3-binding protein	↓	2/4 W	0.44/0.35		
P19804	Nucleoside diphosphate kinase B	↑	4/8 W	2.86/7.24	Borthwick et al. (2016)	
P42123	L-lactate dehydrogenase B chain	↑	4 W	2.67		
P16617	Phosphoglycerate kinase-1	↑	4/8 W	1.62/2.24		
Q9JJ40	Na(+)/H(+) exchange regulatory cofactor-3	↑	4 W	1.51		
O88989	Malate dehydrogenase, cytoplasmic	↑	8 W	2.32	Konga et al. (2009)	
P00758	Kallikrein-1	↑	8 W	2.11	Sexton et al. (2009)	
P07314	γ-Glutamyltranspeptidase K89	↑	8 W	2.05		

This table is cited from Huang et al. (2018)

the development of IPF and contributes to extracellular matrix deposition (Pilewski et al. 2005). Selenoprotein P is upregulated in myofibroblast cultures established from IPF patients, and it maintains the viability of myofibroblasts (Kabuyama et al. 2007). WISP1 was upregulated in alveolar epithelial type II cells of lung fibrosis mice and IPF patients, and WISP1 was identified as a potential therapeutic target of IPF (Konigshoff et al. 2009). Abnormal deposition of extracellular matrix are necessary processes in IPF. In addition, three differential proteins including collagen alpha-1(I) chain, collagen triple helix repeat-containing protein 1, and SPARC-like protein 1 were involved in abnormal deposition of extracellular matrix. These results suggested that urine proteomics has the potential to early diagnosis of IPF and monitor disease progression and reflect treatment response.

Pulmonary function tests are the gold standard for the diagnosis of COPD. However, usually no airway symptoms and airflow limitations appear in the early stages of COPD. Moreover, lungs will be irreversibly damaged in the late stages for which there is no cure (Martinez et al. 2011). This phenomenon is similar to IPF, and early detection and treatment would be an important key toward a more

successful treatment of these disorders. In this chapter, our results showed that urine proteins have the potential to detect respiratory disorders in early disease stages, and early detection may lead to effective treatment for a better prognosis in the lung fibrosis model. In addition, the COPD model and IPF model only have one common differential protein, suggesting urine proteins could be used to differentially diagnose different lung diseases. Our preliminary results show great promise of urinary biomarkers for respiratory disorders. However, urine samples from patients with lung diseases are still needed to validate specific protein patterns as biomarkers for clinical applications.

In addition to lung fibrosis and COPD, urine proteomics has also been applied to other respiratory disorders, such as lung cancer (Zhang et al. 2018), obstructive sleep apnea (Snow et al. 2010), and tuberculosis (Young et al. 2014). These results will provide additional clues for future urine-based biomarker studies involving respiratory diseases. More physicians and researchers should pay more attention to urine biomarkers of respiratory disorders.

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Chapter 14
Application of Urine Proteome in Cardiac Diseases Biomarker Discovery

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Abstract The cardiac disease is a common disorder and is considered as one of the leading causes of death around the world. The early detection of cardiac diseases is urgently needed. Though some diagnostic biomarkers have been used in prediction of diverse cardiac diseases in clinical practice, they are not enough good as expected. The urine proteome studies of cardiac diseases were relatively less than the plasma studies; however, they may provide some useful clues for prediction of the cardiac diseases with the development of high-resolution mass spectrometry technology. In this chapter, we summarize the urinary diagnostic protein biomarker of cardiac diseases (animal models and patients) and review recent urine proteome studies of coronary artery disease, myocarditis, and heart failure. These findings may provide clues for the pathogenesis of diseases, and the results may show that urine is a good source of cardiac diseases biomarkers.

Keywords Urine proteome · Cardiac disease · Biomarker

14.1 Application of Urine Proteome in Studying Cardiac Diseases

The cardiac disease is considered as one of the leading causes of death around the world in the recent years. An estimation of 17.6 million people died from cardiovascular diseases (CVDs) in 2016 accounts for 31% of total deaths worldwide (Benjamin et al. 2017). As a result, the early detection of these diseases and early management of medicine of people with cardiac diseases are urgently needed. Even though some diagnostic biomarkers for acute myocardial infarction, such as troponin I and troponin T, have been widely used, there were still limitations in prediction for the outcome of patients. For other cardiac diseases, few biomarkers with high specificity can be used in clinical practice. A greater understanding of biomarkers of

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the diverse cardiac diseases would help guide the development for the early diagnosis and prognosis of acute events.

As mentioned before in the book, urine proteome has been widely used in the study of numerous diseases; however, it was relatively less utilized in the cardiac diseases than plasma and blood cells (Mokou et al. 2017). It is widely accepted that the cardiac diseases are directly connected with the blood stream, and increased attention has been paid to the area. The clinical approach of diagnosis of cardiac diseases was usually based on symptoms, electrocardiogram, and serum biomarkers. But sometimes it is difficult to differentiate diseases according to the above symptoms and the known biomarkers. As urine was considered a better source for biomarker discovery, previous studies have shown that kidney diseases and even brain diseases can be reflected in urine (An and Gao 2015; Wu and Gao 2015). There were also some urine proteome studies of cardiac diseases that have shown that the urinary biomarkers may reflect the pathologic changes when the cardiac disease occurs.

Different proteomics technologies, including two-dimensional electrophoresis, shotgun proteomics, and targeted proteomics, have been used in biomarker studies. In this chapter, we discuss the urinary diagnostic protein biomarker of cardiac diseases. The proteome-based urine biomarker analyses of diverse cardiac diseases demonstrated that urine may be a good biomarker source of cardiac diseases. A review published in 2017 has demonstrated the urine protein biomarkers for detection of some CVDs in clinic (Rothlisberger and Pedroza-Diaz 2017). In this chapter, the urine protein study of myocarditis based on proteome approach was showed, and some common cardiac diseases will also be discussed (coronary artery disease and heart failure).

14.2 Urine Proteome Changes in Rat Model of Myocarditis

Myocarditis is characterized by inflammation of the myocardium and is triggered by various situations, including autoimmunity and infection (Sagar et al. 2012). As the disease processes, it may result in potentially devastating sequelae like dilated cardiomyopathy (D'Ambrosio et al. 2001). The clinical presentations of myocarditis are heterogeneous, ranging from asymptomatic infection to fatigue, chest pain, arrhythmias, and even heart failure, which increase the difficulty of diagnosis. There were no specific serum biomarkers of myocarditis. And the urine proteome study of myocarditis patients is rarely found. The experimental autoimmune myocarditis (EAM) model is mediated by various types of immune cells and always helps to study the immunological mechanisms of myocarditis (Rose et al. 1993).

Zhao et al. (2018) collected urine at days 0, 14, 21, and 28 from the myosin-induced autoimmune myocarditis rat models and analyzed by using isobaric tandem mass tag labeling approach coupled with two-dimensional reverse-phase liquid chromatography and high-resolution mass spectrometry. A total of 46 urinary proteins were significantly changed in the myocarditis rats, nearly half of them were

reported to be related to myocarditis or be annotated as cardiovascular network. Among the 40 proteins that have human orthologs, 10 were previously reported in serum or cardiac tissue from patients with myocarditis or cardiac injury. Among them, regenerating islet-derived protein 3, cystathionine gamma-lyase, myoglobin, matrix metalloproteinases, heart fatty acid-binding protein, ATP synthase subunit beta, beta 2-microglobulin, and E-selection were previously reported to be related to myocarditis. And they were not the most significantly changed proteins in this study, which may indicate that additional proteins may be candidate biomarkers of cardiac injury in the future.

Also, one of the most significant gene networks that was associated with dysregulated genes in the EAM samples was cardiovascular function. Approximately 40 out of 46 changed proteins were annotated in the GO molecular function analysis, and 10 proteins, such as regenerating islet-derived protein 3, E-selectin, and beta-2-microglobulin, were enriched in immune system processes that play important roles in immune myocarditis development. In addition, the changed proteins were involved in metabolic processes (37%), localization (25%), and cellular processes (23%). Although proteins from all parts of the cell could be detected, extracellular matrix proteins were significantly overrepresented in the main cellular component. This is the first study to use TMT in the urine proteome analysis of EAM; the study showed that urine can be a good source of myocarditis biomarkers.

14.3 Urine Proteome Changes in Common Cardiac Diseases

14.3.1 Coronary Artery Disease

Coronary artery disease (CAD) is the most common type of cardiac diseases and ranks as the leading death of the world (Arzamendi et al. 2011). One common symptom of CAD is an angina. In other words, angina is a common chest pain and results from reduced blood flow to the heart, with an estimation of 2–4% Europeans suffered from the disease (Fox et al. 2006). Although angina is relatively common, the clinical characteristics of angina are not particular and sometimes difficult to distinguish from other chest pains, such as the pain or discomfort of indigestion (Fihn et al. 2012). The urine proteome analysis of angina was usually along with the CAD. When we searched in PubMed website by using the keywords "urine proteomic/proteome; angina," only two studies were listed, and both of them are conducted by CE-MS. From these two studies, some urinary polypeptide patterns were proposed to be close to angina.

Neisius et al. (2016) reported a urine proteome study of patients with angina by using capillary electrophoresis coupled with TOF-MS. A total of 60 patients who underwent elective coronary angiography was included for investigation. They have previously described a panel of 238 urinary polypeptides specific for established

severe coronary artery disease (CAD). For the stale angina, they discovered that the CAD238 score was a significant predictor of the Gensini score that is an indicator of CAD severity. The 238 urinary polypeptides then were considered to have relationship to the CAD burden but are less useful for diagnosis in patients with stable angina if not complemented by other diagnostic tests. It may be concluded that these 238 peptides were not suitable for the diseases that have less extensive coronary artery lesion. In a previous study, (von Zur Muhlen et al. 2009) found 17 urinary polypeptides allowed separation of CAD and non-CAD group with a sensitivity of 81% and a specificity of 92% in 67 patients. And they concluded that these 17 urine peptides may be used for CAD patients with angina-typical symptoms.

Early diagnosis of CAD in the incubation period of coronary heart disease may lead to more effective method for delaying disease progression and play important roles in improving patients' quality of life. More large-scale studies are involved in the urine proteome changes of CAD compared with angina. In an acute coronary syndrome study, urine samples from 252 individuals were enrolled and analyzed by CE-MS (Htun et al. 2017). Among them, 126 patients had suffered from acute coronary syndromes within a period of up to 5 years post urine sampling. A total of 75 urinary peptides combined as a pattern were purposed to have the potential to predict ACS events in patients without obvious signs. The outcome of CAD may be affected by lifestyle choice, and some urine proteome studies revealed that the polyphenol-rich drink may have some beneficial effects on CAD (Mullen et al. 2011).

14.3.2 Heart Failure

Heart failure (HF) is a common and complex syndrome, accompanied with blood filling and/or ejection of the left ventricular dysfunction. Over five million people were diagnosed with HF and the population of getting HF increases year by year (Lloyd-Jones et al. 2010). The disease is a big threat to human health. HF was usually diagnosed when the clinical symptoms are typical, and the patients often require hospital admission. The effective predictive methods of the HF are valuable for establishing optimal treatment. In clinical practice, some serum biomarkers such as NT-proBNP, BNP, and LDL-C have been widely used for assessing the severity of the disease (Han et al. 2015). There are some limitations of BNP such as age and gender difference, vulnerable to potentiation of renal impairment, and relatively broad normal reference range (Fox et al. 2006). As kidney function was usually disrupted after the heart dysfunction, the participants included in the heart failure studies are more important and should be matched for eGFR to avoid bias of renal function (Rossing et al. 2016). As it is difficult to determine that the urine proteome changes were from kidney dysfunction or the heart failure itself, the papers listed

below did not contain the heart failure studies with acute kidney injury or chronic kidney diseases.

For heart failure with reduced ejection fraction, 103 urinary peptides were significantly differentially excreted in 33 patients by online capillary electrophoresis coupled to electrospray ionization micro time-of-flight mass spectrometry (Rossing et al. 2016). These peptides, mainly composed of fibrillar type I and III collagen fragments, fibrinogen beta, and alpha-1-antitrypsin, performed with high sensitivity in discriminating between HF and controls with/without hypertension and renal damage. However, these peptides cannot distinguish diastolic left ventricular dysfunction from the HF with reduced ejection fraction. A similar CE-MS analysis of urine proteome in 49 HF patients was conducted by Zhang et al. (2017). The 96 urinary peptide biomarkers were validated with prognostic value when 17 individuals with asymptomatic diastolic dysfunction progressed to overt HF. For the N-terminal pro b-type natriuretic peptide, the AUC value was as high as 0.7.

For a 2D-DIGE analysis of chronic HF, 15 patients were included. A total of 20 proteins were identified with different expression by MALDI-TOF/MS (Hou et al. 2014). The functional analysis of these changed proteins showed that the alpha-1-acid glycoprotein 1 play central roles in different pathways. Then the ORM1 was validated by Western blot and quantified by ELISA. The authors thought ORM1 could be a potential biomarker for the early detection of CHF. In fact, the ORM1 is a common protein in urine proteome analysis and differentially expressed in many diseases (Shao et al. 2011). The specificity of the only one common urine protein in predicting HF is doubtful.

14.4 Future Perspective

Though urine proteome has been used for CVD studies and has a competitive advantage in some aspects, there are still some limitations: (1) The studies were conducted in a small population or animal models; some valuable changed protein panels should be used for validations in a large population to make it more convincible in the future. (2) For some studies, only one common protein was purposed as a candidate biomarker. As we know, the cardiovascular diseases are complex, and some urine high-abundance proteins in acute phase signaling pathway always changed in many cases. It is very difficult to diagnose a disease only by a common urinary protein. The specificity of these proteins should be considered, and the protein panel may provide more information. (3) The kidney function was usually disrupted after some cardiac diseases happened. As a result, the renal function and the matched eGFR should be taken into accounts before analysis. (4) When we searched published papers in the PubMed website, most of the CVD-related studies including CAD and HF are based on CE-MS. More low-abundance urinary

proteins with high specificity/sensitivity may be found in the future with the development of the MS approaches.

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Chapter 15
Changes of Urinary Proteins in Infectious Disease Models

Yanying Ni and Xundou Li

Abstract Unlike cerebrospinal fluid or blood, urine accumulates metabolic changes of the body and has the potential to be a promising source of early biomarkers discovery. In this chapter, different models were established to mimic the pathophysiological status of infectious diseases, which contributed to explore the significance of urine in biomarkers discovery of infectious diseases. Rat injected with *Escherichia coli* was used to mimic meningitis, and mouse infected with *Plasmodium yoelii* was used to mimic malaria. Some urinary proteins were significantly increased after injection, and most differential proteins were related to inflammation. In another study, several urinary proteins were insignificantly increased in tuberculosis patients' urine, what's more mycobacterial proteins were observed. These important studies laid the foundation to further explore biomarkers of infectious diseases.

Keywords Urine proteome · Early diagnose · Meningitis · Malaria · Tuberculosis · Animal model

15.1 Introduction

Changes are the most fundamental characteristic of biomarkers which reflect changes associated with physiological or pathophysiological processes. Unlike cerebrospinal fluid or blood, urine which is not regulated by homeostatic mechanisms has the potential to be a promising source of early biomarkers discovery (Gao 2013). In this chapter, meningitis model and malaria model were established to search for early diagnostic clues for infectious diseases. Urine were collected before and after injection.

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15.2 Bacterial Meningitis Model

Bacterial meningitis is an important cause of death among neonatal and childhood worldwide (Wijetunge et al. 2014). The incidence of bacterial meningitis in young children is high, and neurological sequelae in the survivors are very common (Kim 2010; Garcia-Hernandez et al. 2016). The clinical symptom, such as fever and chills, is not specific in the acute phase (Baraff et al. 1993). Microbial culture is the gold standard for diagnosis, but this method has low sensitivity (Wu et al. 2013; Taskin et al. 2004). In addition, it is difficult to be widely used in childhood without obvious meningitis symptoms. In order to reduce mortality and morbidity, it's essential to develop a noninvasive method for identifying clues for the early diagnosis of bacterial meningitis.

Changes in urinary proteins can be affected by various physiological and pathological factors, including sex, age, medication, and so on (Wu and Gao 2015). Rats infected with *Escherichia coli* were used to mimic the pathophysiological status of bacterial meningitis in children (Liu et al. 2012). These rats were randomly divided into two groups with nine rats in each group. The bacterial meningitis model was introduced as described previously (Leib et al. 2000). The meningitis group was injected with 50 μl of *Escherichia coli*, and control group was injected with saline. All rats were scored clinically at the 1st and 3rd day after injection (Leib et al. 2001).

All rats were sacrificed and their brains were fixed. Clinical observation found that meningitis group had obvious inflammation (Fig. 15.1). Blood vessels in the subarachnoid and brain parenchyma exhibited marked dilation and hyperemia.

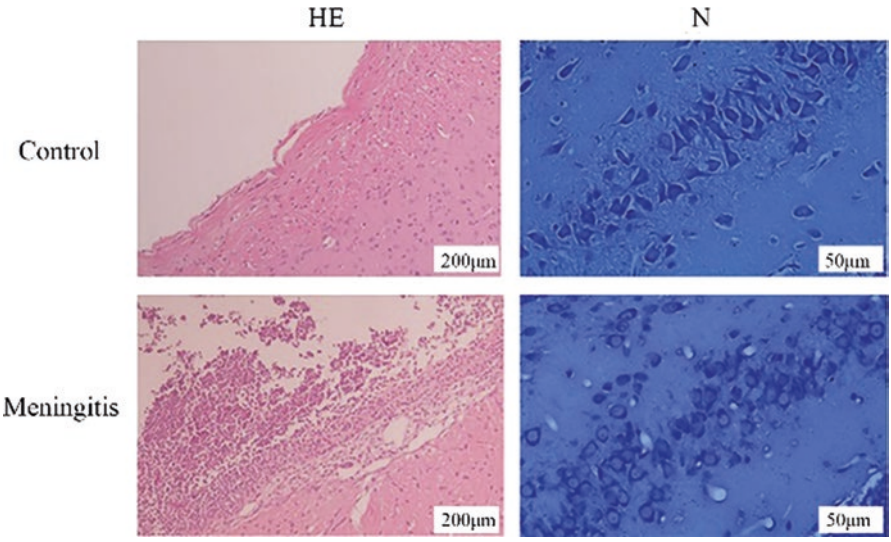

Fig. 15.1 Histopathology of brain tissue from rats with meningitis

The control group showed neither meningeal inflammation in the subarachnoid space nor damage in the hippocampal neuron. The pathological results showed that the model was successfully established.

After the injection of *Escherichia coli*, the rats in meningitis group experienced tachypnea, decreased feeding, spontaneous activity, and drowsiness, and had low symptom scores, while the control group did not show visible neurological symptoms (Fig. 15.2a). All subjects were weighed before and after injection (Fig. 15.2b). Compared with the control group, the weight of meningitis group declined significantly after 24 h, and this trend lasted for 4 days.

The urinary proteins at the 1st and 3rd day were analyzed by SDS-PAGE (Fig. 15.3). Among the meningitis group's proteins at the 1st day, the expression of the 170 and 15 kDa bands were decreased, and 130, 55, and 35 kDa bands were increased. However, these obvious differences disappeared at the 3rd day.

The urine proteins at the 1st day were digested by two methods, including in-gel digestion and filter-aided digestion. Five obvious bands were selected to identify differentially expressed proteins. The urine proteins at the 3rd day which do not have significant differential band were digested by filter-aided digestion. Urine proteins were analyzed by Thermo Orbitrap Fusion Lumos.

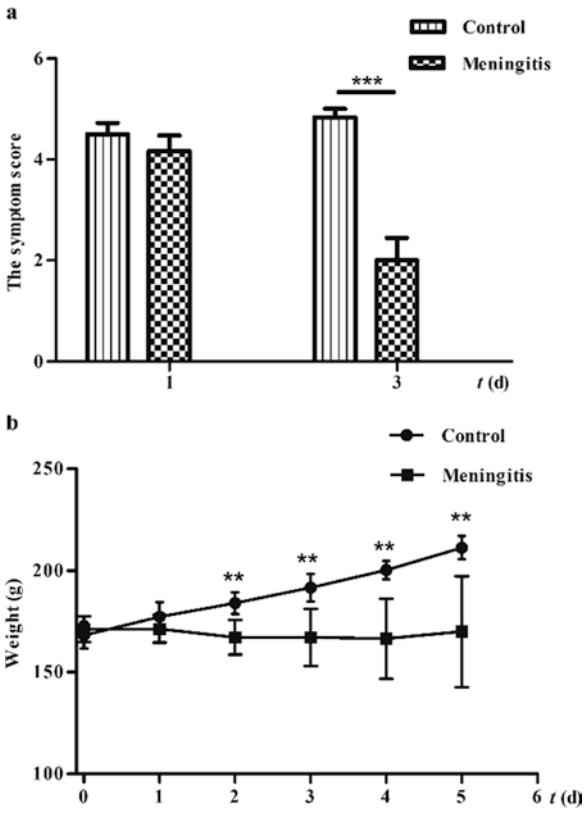

Fig. 15.2 Clinical parameters of rats in the meningitis. (**a**) The symptom scores of rats on day 1 and 3 after the injection. (**b**) The weight of experimental rats

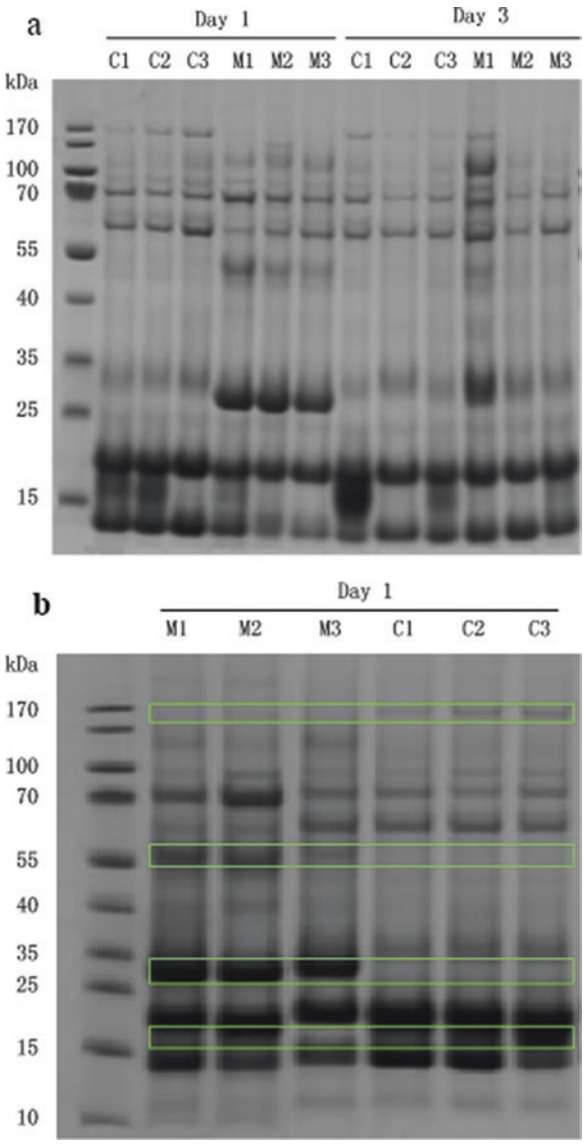

Fig. 15.3 SDS-PAGE analysis of urinary protein samples. C1-3, samples from the control group; M1-3, samples from the bacterial meningitis group. (**a**) The urinary proteins of the 1st and 3rd day after surgery. (**b**) Five clear differential bands of the 1st day's urinary proteins

It is showed that 17 differentially expressed proteins were identified at the 1st day's urine of all groups, which was digested by the in-gel digestion (Table 15.1). Among them, 11 differential proteins were increased. In addition, 20 differential proteins were identified by the filter-aided digestion method (Table 15.2). In these, 11 were increased. Some proteins, alpha-1-acid glycoprotein (A1AG), alpha-1-antiproteinase (A1AT), neutrophil gelatinase-associated lipocalin (NGAL), T-kininogen 1 (KNT1), and pro-epidermal growth (EGF), were identified in two results. Through the filter-aided digestion method, five differential proteins were

Table 15.1 Differential expressed proteins between the control group and bacterial meningitis in-gel digestion

Protein name	*P* value	Fold change	Spectral counts					Trend	
			C1	C2	C3	M1	M2	M3	
170 kDa									
Pro-epidermal growth factor	0.006	0.148	134	168	96	15	29	15	↓
Alpha-1-inhibitor 3	0.018	2.972	14	9	13	33	47	27	↑
Galectin-3-binding protein	0.040	2	8	5	4	9	11	14	↑
55 kDa									
Fibronectin	0.040	0.654	21	15	19	12	10	14	↓
Alpha-1-acid glycoprotein	0.022	2.733	13	7	10	34	29	19	↑
T-kininogen 1	0.01	4.375	4	3	1	15	11	9	↑
Matrix-remodeling-associated protein 8	0.017	0.426	26	19	23	5	10	14	↓
35 kDa									
Alpha-1-antiproteinase	0.004	26	2	0	0	19	12	21	↑
Alpha-2HS-glycoprotein	0.038	0.413	7	13	9	3	4	5	↓
Prostatic spermine-binding protein	0.006	3.142	2	3	2	7	6	9	↑
Glutathione S-transferase alpha-1	0.032	0.300	7	9	14	2	3	4	↓
Apolipoprotein M	0.009	0.208	7	10	7	0	2	3	↓
Neutrophil gelatinase- associated lipocalin	0.000	16.38	7	6	5	99	94	102	↑
15 kDa									
Serum albumin	0.021	6.733	5	0	10	48	26	27	↑
Serotransferrin	0.033	17	0	0	2	16	5	13	↑
Serine protease inhibitor A3L	0.001	4.363	2	3	6	17	15	16	↑

C and M represent the control group and meningitis group, respectively, with C1 and M1 representing No. 1 rat in each group

detected, and two out of five were increased. The trends for the expression of complement C4 were consistent at the 1st and 3rd day. Among all the differential proteins, 16 of the 18 were human orthologous proteins in the Ensembl homolog database (Jia et al. 2013).

The differential proteins were analyzed by the PANTHER classification system. It's helpful to study the molecular function, different biological processes, and distribution information. The analysis showed that the same differential protein had different molecular functions and could be involved in different biological processes. For example, ceruloplasmin was not only involved in different biological processes, such as combine, catalyze, acceptor, and transport activity, but also participated in localization, metabolism, and multicellular organisms.

The changes of urinary proteome may come from different pathways. Urinary differential proteins may inherit from CSF or blood, and they may also be generated within the body pathophysiological processes, such as infection and stress. In this study, the meningitis group had lower urine protein-to-creatinine ratios at the 1st day. However, the trend was reversed at the 3rd day (Fig. 15.4), which suggests the renal function of meningitis group was not significantly different with the control

Table 15.2 Differential expressed proteins between the control group and bacterial meningitis in filter-aided digestion group

Protein name	*P* value	Fold change		Spectral counts			Trend
				C	M1	M3	
Alpha-1-macroglobulin	0.00	0.67		101	67		↓
Low-density lipoprotein receptor-related protein 2	0.02	0.67		93	62		↓
Pro-epidermal growth factor	0.00	0.64		98	63		↓
Alpha-1-acid glycoprotein	0.00	4.12		21	87		↑
Alpha-1-antiproteinase	0.01	1.83		29	54		↑
T-kininogen 1	0.00	1.96		29	57		↑
Neutrophil gelatinase-associated lipocalin	0.00	3.08		13	41		↑
Haptoglobin	0.02	2.68		12	31		↑
Ceruloplasmin	0.02	2.17		13	27		↑
Cathepsin B	0.01	1.60		8	14		↑
Beta-2 microglobulin	0.00	2.64		4	10		↑
CD48 antigen	0.00	0.52		7	4		↓
Complement component C9	0.03	2.04		4	9		↑
Protein FAM151A	0.00	0.44		5	2		↓
Cathepsin S	0.01	1.71		3	6		↑
Biglycan	0.00	0.20		6	1		↓
Attractin	0.04	0.52		5	3		↓
Nidogen-2	0.02	0.64		6	4		↓
Putative phospholipase B-like 2	0.03	0.53		5	2		↓
Serine protease inhibitor A3M	0.00	0.61		9		5	↓
Lysosomal alpha-glucosidase	0.01	0.40		7		3	↓
Carboxypeptidase Q	0.04	1.50		15		22	↑
Complement C3	0.02	0.62		21		13	↓
Complement C4	0.00	1.83	1.85	9	16	16	↑

C represents the control group, and M represents the meningitis group, respectively, with M1 and M3 representing day 1 and day 3

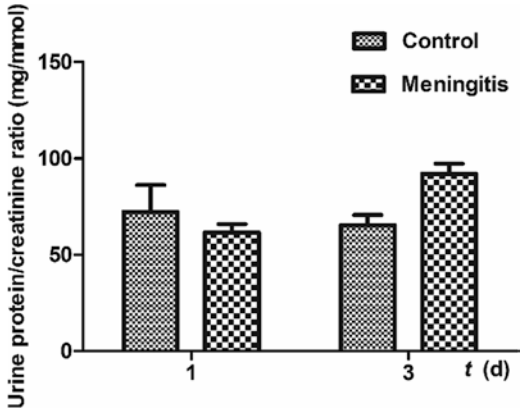

Fig. 15.4 The analysis of the urine protein-to-creatinine ratio (Up/Ucr). Compared with control rats, the Up/Ucr of meningitis rats was higher on the first and lower on the third day after bacterial implantation. However, the differences were not statistically significant

group. The above shows that urinary differential proteins were derived from changes in CSF or blood, and the urinary proteome will change in brain diseases.

A1AG and A1AT, which play a major role in modulating the activity of the immune system during the acute-phase reaction, were increased in the CSF and blood in patients with bacterial meningitis (Paradowski et al. 1995). Serotransferrin, which is involved in metabolic responses, is also increased in the blood of patients with bacterial meningitis (Caksen et al. 2003). Galectin-3-binding protein, beta2-microglobulin, and neutrophil gelatinase-associated lipocalin are increased in patients' CSF with bacterial meningitis (Nasioudis and Witkin 2015; Bellac et al. 2007; Hansen et al. 1992). Complement C4, which may be involved in the body's response to irritation, is downregulated in the cerebrospinal fluid of patients (Oren et al. 1995). The level of fibronectin in the patients' CSF is significantly elevated (Weller et al. 1990). Most of these differential proteins can reflect the inflammation infection and immune stress in the body.

15.3 Mouse Malaria Model

In the above study, the rat injected with *Escherichia coli* was used to mimic bacterial meningitis, which mainly associated with inflammation and immune. Human malaria is an infectious disease with a high mortality rate (Snow et al. 2005; Hay et al. 2010), mostly among children (Bryce et al. 2005). It is urgent to search for early diagnostic clues.

The current diagnostic methods for malaria are mainly based on blood and mitochondrial PCR-based malaria test (Ghayour Najafabadi et al. 2014; Oguonu et al. 2014; Oyibo et al. 2017). Urinary differential proteins provide early diagnostic clues for bacterial meningitis, a contagious brain disease. Maybe, it is also a better method to diagnose malaria. In this study, mouse injected with lethal *Plasmodium yoelii* was used to mimic malaria (Shao et al. 2010). The target of this study was attempted to search for malaria-associated protein in the urine of malaria mice.

Mice were randomly divided into six groups, and urine was collected before and after infection. Urine samples at the 4th and 5th day were processed by in-gel digestion. The samples at the 4th day were analyzed by Triple TOF 5600 MS system. Peptides information were searched in the Plasmodium yoelii, vivax and falciparum databases to identify the Malaria-associated proteins, but no reliable protein was found.

Urine samples at the 5th day were analyzed using a better mass spectrometer at present, the Orbitrap Fusion Lumos MS system, which yielded the same result. However, the identification depth was further improved with 821 urinary proteins detected.

In this study, there were other results that 21 and 83 differential urinary proteins were detected at the 4th and 5th day, respectively. Four differential proteins (Ig alpha, galectin-3-binding protein, mucin-2, apolipoprotein D), which have been

reported to be related with infection (Ganfornina et al. 2008), were significantly increased after injection.

15.4 Other Infectious Disease Models

Under the current depth of mass spectrometry, no parasite proteins were identified in malaria urine. In another study, pathogen-associated proteins were identified in patients' urine (Young et al. 2014). Tuberculosis (TB) is a major global health problem. In their study, patients were divided into three groups: definite TB, presumed latent-TB infection (LTBI), and presumed non-TB/non-LTBI. Urine samples were also processed by in-gel digestion. They used MWCO filters to remove the higher molecular weight human proteins, such as uromodulin and albumin, which enabled them to identify and quantify on average 560 proteins. Compared with the non-TB/non-LTBI group, the TB group had upregulated levels of immunoglobulin kappa chain C, retinol-binding protein 4, A1AG, and immunoglobulin lambda-2 chain C. What's more, mycobacterial proteins were observed in the urine of definite TB group and presumed LTBI group, which lays the important foundation for urine as a biomarker resource for infectious diseases.

Some differential proteins which were identified in meningitis rats had been also reported in other studies. EGF was downregulated in the urine of Fanconi syndrome (Cutillas et al. 2004), A1AT was increased in the urine of diabetes (Sharma et al. 2005), and A1AG was increased in acute appendicitis and uremia (Watson et al. 2012; Vasson et al. 1993). Galectin-3-binding protein, which was increased in meningitis' urine, was also increased in malaria urine. It is possible that these proteins are differentially expressed in many other diseases which have the same pathophysiological processes, including inflammation and stress. Thus, a panel of different proteins involved in different pathophysiological processes is more likely to reflect changes in the body.

A comparison of mass spectrometry results between the in-gel digestion and filter-aided digestion, which are from the 1st day urine, showed that some proteins were repeatedly identified while others were different. The traditional top-down proteomic strategy will provide more detailed information. However, it is difficult to distinguish an intact protein with degraded fragments. Instead, the proteomic protocol based on 1D SDS-PAGE and in-gel digestion circumvents this issue. For example, serotransferrin (76 kDa) was identified on the 15 kDa band. It's the reason that we identified one of the degraded fragments of serotransferrin. Alpha-1-acid glycoprotein (24 kDa) appeared on the 55 kDa band. A possible reason is that the protein had been modified, such as glycosylation, which leads to an increase in molecular weight. Thus, before filter-aided digestion, samples should first be analyzed by 1D SDS-PAGE. If there are differential bands between the control and experimental groups, samples should be analyzed using a comprehensive method that includes in-gel and filter-aided digestion.

Urinary differential proteins may have different source. One way is cerebrospinal fluid or blood; another way is damaged kidneys which is caused by pathophysiological process, such as infection and stress. In addition to urinary system diseases, the urinary differential protein could also reflect other system diseases.

The objective of above studies is to analyze the urinary proteome changes of infectious diseases. The proteomic results showed it is after the infection that the urinary proteome had significant changes. In future biomarker discovery studies, with the development of proteome analysis, more and more low abundance urinary proteins can be identified, which will provide more valuable diagnostic clues.

And if the pathway and function of urinary differential proteins in infectious diseases can be further studied, a better relationship will be built between differential proteins and the pathophysiology process of infectious diseases.

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Chapter 16
The Application of Urinary Proteomics in Early Detection of Digestive Diseases

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Abstract Digestive diseases are disorders of the digestive organs, which may range from benign to serious. There is an inherent difficulty in diagnosis and treatment of various chronic digestive diseases; thus noninvasive early biomarkers are desperately needed. Without homeostasis mechanisms, urine is an ideal biomarker source that can reflect the early changes of disease theoretically. In this section, we will introduce the new studies of the urine proteome in rat models of three digestive diseases including liver fibrosis, chronic pancreatitis and inflammatory bowel disease. Many potential biomarkers were found earlier than clinical symptom and significant pathological changes that may provide important clues for the early detection of these diseases. We think urine proteome has a broad application prospect in the early diagnosis, treatment, monitoring, and prognosis of digestive diseases.

Keywords Urinary proteomics · Liver fibrosis · Chronic pancreatitis · Inflammatory bowel disease · Animal models

16.1 Introduction

As the main function of the digestive system, digestion is important for the body to obtain energy and sustain life. Digestive diseases refer to the acute or chronic diseases of digestive organs mainly including the esophagus, stomach, intestine, liver, gallbladder, and pancreas. The incidence of digestive diseases showed an increasing trend, which is a serious threat to human health and life. Also, chronic injury of digestive organs is a risk factor for the development of cancers. However, there are

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challenges in the clinical early diagnosis of chronic digestive diseases, even though many new diagnostic treatment technologies have greatly promoted the diagnostic level (Meng et al. 2015). Therefore, it is urgent to discover noninvasive biomarkers for early preclinical diagnosis.

Urine accumulates early changes of the body, which makes it a better early biomarker source (Gao 2013). Mass spectrometry (MS)-based proteomics has dramatically improved and emerged as a prominent tool in the field of biomarker study. Urinary proteomics has become increasingly important in studies of quantitative changes in proteins resulting from disease and used to explore the pathogenesis or identify potential biomarkers of diseases (Jing and Gao 2018). Animal models can be used to minimize the impact of many uncertain factors by establishing a direct relationship between a disease and corresponding changes in urine. Here, the proteomic analysis in urine from three digestive disease rat models were performed using mass spectrometry to identify the potential biomarkers for future clinically noninvasive and early diagnosis.

16.2 The Study of Digestive Disease in Animal Models Through Urinary Proteomics

16.2.1 Liver Fibrosis

Liver fibrosis is a state of liver injury in which excessive extracellular matrix (ECM) components, especially collagen, are deposited and disturb normal liver functions, which results in many chronic liver diseases (Friedman 2008; Wynn 2008). During the process of chronic liver injury, fibrotic scar tissue is gradually formed due to excessive deposition, hepatic architecture is distorted, and nodules of regenerating hepatocytes are ultimately generated, which result in cirrhosis (Guo and Friedman 2007; Tennakoon et al. 2015). Liver fibrosis is a reversible pathological condition, whereas cirrhosis, the end stage of liver fibrosis, is irreversible (Popper and Kent 1975; Tangkijvanich and Yee 2002). Cirrhosis can affect the risk of developing primary liver cancer or hepatocellular carcinoma (HCC) (Seow et al. 2001). For now, liver puncture biopsy is still the gold standard for the diagnosis of liver fibrosis. Consequently, noninvasive biomarkers that have adequate specificity and sensitivity and respond quickly to changes in the fibro genic process are desperately needed (Guo and Friedman 2007).

Among the liver fibrosis animal models, the intraperitoneally TAA-injected rat model is widely used to induce liver fibrosis and cirrhosis and consistently produces liver fibrosis and cirrhosis in rats with a histopathology that is more similar to that of human liver fibrosis and cirrhosis (Noda et al. 1996; Li et al. 2002; Aydin et al. 2010). Male Sprague-Dawley rats were injected intraperitoneally with 200 mg/kg

TAA thrice weekly for 8 weeks to establish the liver cirrhosis model, and the control group was replaced with saline. The urine samples were collected at weeks 1, 3, 6, and 8. At weeks 6 and 8, there were a significant increase in the serum levels of liver function biomarkers alanine aminotransferase (ALT) and aspartate transaminase (AST), and a significant decrease in the serum levels of total protein (TP) and albumin(ALB) compared to those in the respective control groups, indicating hepatocyte damage (Zhang et al. 2018a). At weeks 1 and 3, no obvious histological changes of liver tissue were observed under microscope. At week 6, some inflammatory cells, collagen deposition, and hydropic degeneration of endothelial cells and hepatocytes were observed mainly in the centrilobular areas forming thin fibrous septa around the central veins, whereas the hepatic lobules were almost well-arranged. At week 8, fibrous bridges were completely formed between the central veins and the central veins to the portal areas, thus separating the liver parenchyma into a typical pseudo-lobule. The fibrous bridges became thicker, resulting in complete cirrhosis (Fig. 16.1).

The urinary proteomes of rats exposed to TAA for 1 and 3 weeks were profiled using the tandem mass tag (TMT) labeling approach coupled with liquid chromatography and high-resolution mass spectrometry (LC-MS/MS). 143 and 118 significantly changed urinary proteins were identified relative to control at weeks 1 and 3, respectively, with 90 proteins overlapping between these two sets of significantly changed proteins. All of the significantly changed proteins met the following criteria: (1) the proteins had at least two unique peptides, (2) the variation trend of the proteins in each rat was consistent, and (3) the fold change was >2 and p value <0.05. From the significantly changed urinary proteins, 51 proteins exhibited the same average trend in the differential abundance of the proteins at weeks 1, 3, 6, and 8 observed by both high-throughput analysis and the multiple reaction monitoring (MRM) targeted proteomics. Moreover, 40 proteins were statistically significant ($P < 0.05$) with a fold change greater than two according to their abundance among groups, strongly supporting their potential clinical relevance in liver fibrosis (Table 16.1) (Zhang et al. 2018a).

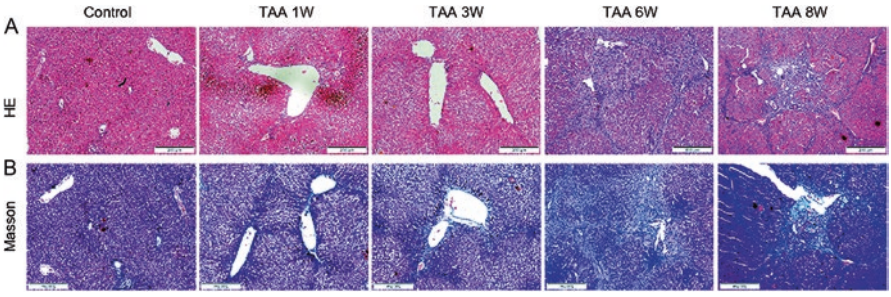

Fig. 16.1 Pathological morphologies of the liver in the control and TAA groups. (**a**) Hematoxylin and eosin stain (HE) staining; (**b**) Masson's trichrome staining (Zhang et al. 2018a)

Table 16.1 Details of changed urinary proteins identified in the TMT method (Zhang et al. 2018a)

Protein name	Uniprot	Human uniprot	FC 1w	P value 1w	FC 3w	P value 3w	FC 6w	P value 6w	FC 8w	P value 8w	Biomarker	Mechanism
Uteroglobin	P17559	P11684	13.62	0.028	34.59	0.0104	50.03	0.0001	127.69	0.028		
Neutrophil gelatinase-associated lipocalin	P30152	P80188	20.01	0.0044	6.79	0	134.56	0	27.39	0.0001	Ariza et al. (2015, 2016) and Russ et al. (2015)	
D-dopachrome decarboxylase	P80254	P30046	52.91	0.0338	10.72	0.007	40.63	0.0031	15.51	0.0007		
Torsin-1A-interacting protein 2	Q6P752	Q8NFQ8	18.13	0.029	13.55	0.0009	24.51	0.0003	30.16	0.0032		
Complement factor D	P32038	P00746	10.95	0.0008	10.51	0	15.04	0.0009	27.92	0.0027		
Protein disulfide-isomerase A3	P11598	P30101	16.87	0.0183	5.1	0	9.14	0	19.53	0.0002	Zhang et al. (2015)	
Alpha/beta hydrolase domain-containing protein 14B	Q6DGG1	Q96IU4	36.05	0.0045	2.76	0.0057	4.76	0.0008	3.54	0.0022		
Ribonuclease 4	O55004	P34096	10.18	0.0003	13.67	0	13.17	0.0082	8.2	0.0005		
Mannan-binding lectin serine protease 2	Q9JJS8	O00187	9.4	0.0043	8.04	0.0028	14.36	0.0325	12.69	0.0248		El Saadany et al. (2011)
3-mercaptopyruvate sulfurtransferase	P97532	P25325	9.06	0.0004	4.93	0	12.42	0.0016	17.94	0		Mani et al. (2014)
Superoxide dismutase [Cu-Zn]	P07632	P00441	12.86	0.0059	6.2	0	15.96	0	8.51	0.0026	Wang et al. (2013)	
Carbonic anhydrase 3	P14141	P07451	6.32	0.0003	3.61	0	4.9	0.0001	25.36	0.0001	Carter et al. (2015)	Dai et al. (2008) and Chai et al. (1991)
Serotransferrin	P12346	P02787	4.43	0.0077	3.17	0.0125	8.41	0.003	21.94	0	Cho et al. (2014)	

Ketohexokinase	Q02974	P50053	18.41	0.0006	3.96	0.0001	11.47	0.0032	4.09	0.0003	Tan et al. (2010) and Gluckmann et al. (2007)	
Protein DJ-1	O88767	Q99497	13.08	0.0007	4.14	0.0001	12.04	0.0043	8.14	0.0004	Liu et al. (2010)	
Putative lysozyme C-2	Q05820	P61626	11.01	0.0003	7.81	0.0166	9.08	0.0196	8.69	0.0271		
Serum albumin	P02770	P02768	6.49	0.0004	3.21	0.0023	8.31	0.0003	18.32	0		
Mesothelin	Q9ERA7	Q13421	10.45	0.0129	5.89	0.0002	9	0.0066	10.39	0.0048		
Secreted phosphoprotein 24	Q62740	Q13103	9.16	0.0002	3.99	0	12.49	0.0001	8.5	0.0011		
Thioredoxin	P11232	P10599	7.93	0.0097	5.57	0.0037	8.1	0.0001	11.76	0.0004	Okuyama et al. (2005)	Okuyama et al. (2005, 2008)
T-kininogen 1	P01048	Null	11.18	0.0001	3.77	0	7.9	0.0003	9.55	0	Henkel et al. (2011)	
Extracellular superoxide dismutase [Cu-Zn]	Q08420	P08294	5.05	0.0103	2.79	0.0003	7.7	0.022	14.19	0.0069		
Cystatin-C	P14841	P01034	11.28	0.0137	4.26	0	5.31	0	8.02	0	Chu et al. (2004) and Ladero et al. (2012)	
Lumican	P51886	P51884	4.81	0.025	3.34	0.0216	6.4	0.003	13.87	0.0099	Bracht et al. (2015)	Krishnan et al. (2012)
Carboxylesterase 1C	P10959	Null	6.41	0.0309	4.18	0.0002	8.34	0.0011	9.46	0		
Cathepsin Z	Q9R1T3	Q9UBR2	9.46	0.0102	6.62	0	7	0.0001	5.06	0		Wang et al. (2011)
Regenerating islet-derived protein 3-gamma			5.86	0.0084	7.85	0.0006	5.64	0.0112	8.76	0.0001		
Alpha-1-antiproteinase	P17475	P01009	7.36	0	5.11	0	11.18	0.0009	4.25	0.0075	Mondal et al. (2016)	Mondal et al. (2016) and Topic et al. (2012)

(continued)

Table 16.1 (continued)

Protein name	Uniprot	Human uniprot	FC 1w	P value 1w	FC 3w	P value 3w	FC 6w	P value 6w	FC 8w	P value 8w	Biomarker	Mechanism
Cathepsin L1	P07154	P07711/ O60911	7.22	0.0198	4.29	0.0004	9.37	0.0003	5.28	0.0006		
Nidogen-2	B5DFC9	Q14112	5.06	0.0003	5.52	0	10.68	0	2.92	0.0096	Cheng et al. (2012)	Cheng et al. (2012)
Cadherin-17	P55281	Q12864	5.42	0	4.66	0.0003	3.58	0.0052	8.5	0	Lee et al. (2010)	Lee et al. (2010) and Su et al. (2008)
CD166 antigen	O35112	Q13740	4.23	0.0097	3.79	0	6.71	0.0003	6.91	0.0001	Ma et al. (2015)	
Ig gamma-2B chain C region	P20761	Null	5.15	0.0284	2.34	0.0491	6.57	0.0028	7.12	0.024		
Protein AMBP	Q64240	P02760	4.8	0.0065	5.09	0.0003	6.8	0.0001	3.94	0.0285		
Serine protease inhibitor A3M (Fragment)	Q63556	P01011	3.73	0.0243	2.01	0.001	4.94	0	9.34	0		
Acidic mammalian chitinase	Q6RY07	Q9BZP6	5.99	0.0002	4.49	0	3.62	0.0002	4.78	0		
Collectin-12	Q4V885	Q5KU26	4.81	0.0033	3.85	0	4.24	0.0001	5.43	0.0004		
EGF-containing fibulin-like extracellular matrix protein 1	O35568	Q12805	3.78	0.0035	3.56	0	4	0.0001	6.39	0.0001		
Multiple inositol polyphosphate phosphatase 1	O35217	Q9UNW1	5.44	0.0104	3.54	0.0007	3.37	0.0001	3.08	0		
Ephrin-B1	P52796	P98172	2.8	0.0099	2.83	0	2.97	0.0001	4.99	0		Sawai et al. (2003)

FC, fold change; Biomarker, proteins were reported as the biomarkers of liver fibrosis, cirrhosis, or other related diseases; Mechanism, proteins make definite effects in the pathologic mechanism of liver fibrosis

Among the 51 changed proteins, some had previously been reported as biomarkers of liver fibrosis, cirrhosis, or other related diseases and were listed in Table 16.1. For example, the expression level of ketohexokinase is clearly upregulated in mice with Con A-induced hepatitis and is one of the potential hepatocarcinogenic biomarkers (Tan et al. 2010; Gluckmann et al. 2007). Protein DJ-1expression is significantly upregulated in hepatocellular carcinoma (HCC), and its expression level correlates with clinicopathological variables and prognosis of HCC patients, which suggests that DJ-1 may be a candidate prognostic biomarker of HCC (Liu et al. 2010). Neutrophil gelatinase-associated lipocalin is a urine biomarker of acute-on-chronic liver failure and cirrhosis (Ariza et al. 2015, 2016; Russ et al. 2015).

Some of the significantly changed proteins identified here have been reported to relate to the development of liver fibrosis or other related diseases. Overexpression of cathepsin Z contributes to tumor metastasis by inducing epithelial mesenchymal transition in HCC (Wang et al. 2011). Ephrin-B1 may be involved in in vivo tumor progression by promoting neovascularization in HCC (Sawai et al. 2003). Mannan-binding lectin-associated serine protease activates human hepatic stellate cells, which is activated in the pathogenesis of liver fibrosis (El Saadany et al. 2011). 3-mercaptopyruvate sulfurtransferase (MST) is expressed in the liver and regulates liver functions via H_2S production, and malfunction of hepatic H_2S metabolism may be involved in many liver diseases, such as liver fibrosis and liver cirrhosis (Mani et al. 2014).

What's more, several proteins have been previously reported to be associated with the pathology and mechanism of liver fibrosis, cirrhosis, and other related diseases as well as the biomarkers of these diseases, including carbonic anhydrase 3, thioredoxin, lumican, alpha-1- antiproteinase, nidogen-2, and cadherin-17. For example, carbonic anhydrase 3 promotes transformation and invasion capability in hepatoma cells through FAK signaling pathway and is a major participant in the liver response to oxidative stress (Dai et al. 2008; Chai et al. 1991). Carbonic anhydrase 3 is also a biomarker of liver injury (Carter et al. 2015). Thioredoxin has a potential to attenuate liver fibrosis via suppressing oxidative stress and inhibiting proliferation of stellate cells and plays important roles in the pathophysiology of liver diseases (Okuyama et al. 2005, 2008). Overexpression of thioredoxin has been reported to prevent TAA-induced hepatic fibrosis in mice (Okuyama et al. 2005). Lumican is a prerequisite for liver fibrosis, and the altered expression of lumican has been associated with liver fibrosis (Bracht et al. 2015; Krishnan et al. 2012). There were also some differential proteins discovered that have never been reported to relate to liver fibrosis, such as torsin-1A-interacting protein 2, putative lysozyme C-2, and cathepsin L1. Since these proteins were changed dramatically, they also have the potential to be early urinary biomarkers of liver fibrosis.

16.2.2 Chronic Pancreatitis

Chronic pancreatitis (CP) is an irreversible bio-inflammatory disease that eventually leads to loss of exocrine and endocrine function, causing complications such as steatorrhea, loss of weight, and diabetes. The main histologic features of CP are chronic inflammation and interstitial fibrosis of the pancreas (Majumder and Chari 2016). There is no widely used diagnostic standard for CP, and early diagnosis is difficult. Abdominal pain is the main clinical symptom, but this is not specific to CP. Imaging and pancreatic function tests are of limited value in the early diagnosis of the disease (Duggan et al. 2016). At present, treatment of CP can only temporarily relieve pain and complications but cannot cure the disease (Ito et al. 2016). Meanwhile, chronic inflammation of the pancreas in CP is a risk factor for the development of pancreatic cancer (PC) (Raimondi et al. 2010). If diagnosed earlier, effective measures may be used to prevent the development of cancer.

Oxidative stress is an important factor in the development of pancreatic fibrosis (Bhardwaj and Yadav 2013). Diethyldithiocarbamate (DDC) can inhibit superoxide dismutase activity, thereby increasing lipid peroxides, causing progressive pancreatic injury and repeated oxidative stress leading to pancreatic fibrosis. Repeated administration of DDC to cause pancreatic fibrosis produces a model of CP with similar pathological process and pathological features to those of human CP (Matsumura et al. 2001). Male Wistar rats were given an intraperitoneal injection of 500 mg/kg body weight of DDC twice per week for 6 weeks. There were no obvious pathological changes in the pancreas as compared with control at week 2. Slight inflammatory cytokines infiltration, acinar disruption, and collagen deposition appeared in some sections of pancreatic tissues at week 3. At week 4 and week 6, pancreatic histologic changes under microscope showed the atrophy of acini, interstitial edema with large amount of infiltrating inflammatory cells, and increased accumulation of collagen fibers (Fig. 16.2).

The urinary proteome in DDC-induced CP rats was analyzed by LC-MS/MS to screen the differential proteins associated with disease progression and find some clues for early biomarkers of CP. By label-free quantitative and statistical analyses, 50 differential proteins that had human orthologs were identified and significantly changed in all three rats (fold change ≥ 2 or ≤ 0.5, $P < 0.05$, Table 16.2). Among these proteins, 15 differential proteins were significantly changed when no obvious pathological changes had yet appeared at week 2. Five of these differential proteins were associated with CP or pancreatic-related diseases. β-2-microglobulin (B2M), which is an important diagnostic and prognostic serum tumor marker, has been reported as increased in the urine and serum of CP patients (Pezzilli et al. 1995). Serum amyloid P-component (SAMP) was also reported as differentially changed in the serum of CP patients and may be a potential serum marker that differentiates PC from CP (Saraswat et al. 2017). COMP was reported to be a marker for tissue destruction and disease activity in CP, with COMP being preferentially expressed in degenerating acinar cells in CP and in CP-like areas in PC (Liao et al. 2003). REG3G

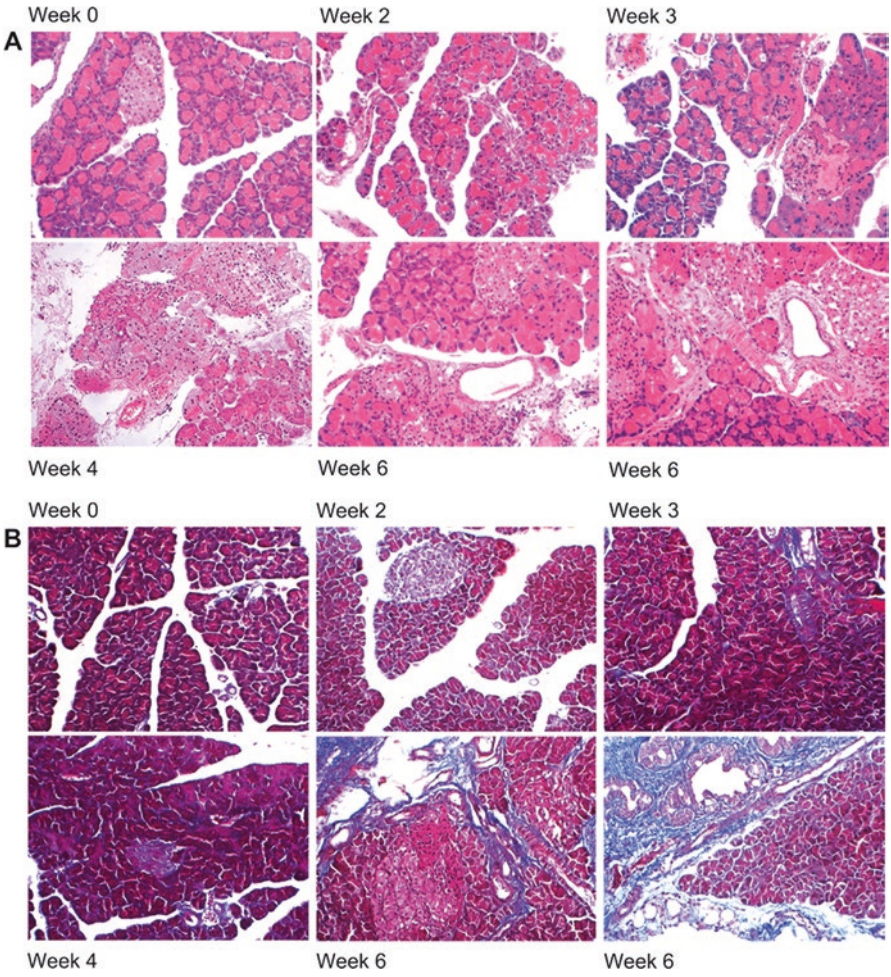

Fig. 16.2 Histopathological characterization of the pancreatic tissue in DDC-induced CP rats. (**a**) HE staining (200×); (**b**) Masson's trichrome staining (200×) (Zhang et al. 2018b)

is a key element in the inflammation of CP, and enhanced expression of REG3G promotes pancreatic carcinogenesis in a murine model of CP (Yin et al. 2015). Interleukin-4 receptor subunit alpha (IL4RA) was overexpressed in human solid tumors, such as those in PC. Meanwhile, IL-4Ra signaling is required for pancreatic fibrosis and alternative activation of macrophages in CP (Xue et al. 2015).

Among the differential proteins detected at the stages of week 3 and week 4, 14 urinary proteins had been reported to be differentially expressed in the serum or pancreatic tissue of chronic pancreatitis patients and other animal model studies. For example, CSPG4 was decreased in the serum and was upregulated in the tissue mRNA levels of CP patients (Keleg et al. 2014). THY1 plays an important role in

Table 16.2 Differential urinary proteins in DDC-induced CP rats (Zhang et al. 2018b)

Accession	Protein name	Fold change				*P* value	Reported to be related to pancreatic disease
		W0	W2	W3	W4		
P07151	Beta-2-microglobulin (B2M)	1	2.87	3.83	3.77	5.40E-05	Serum Pezzilli et al. (1995)
P07897	Aggrecan core protein (PGCA)	1	0.47	0.37	0.41	6.10E-05	
P25113	Phosphoglycerate mutase 1 (PGAM1)	1	0.5	0.37	0.4	3.10E-03	
Q9EPB1	Dipeptidyl peptidase 2 (DPP2)	1	0.49	0.34	0.38	1.10E-05	
Q6AYD4	Endothelial cell-selective adhesion molecule (ESAM)	1	0.47	0.37	0.36	1.70E-02	
B5DFC9	Nidogen-2 (NID2)	1	0.43	0.33	0.34	1.00E-05	
P23680	Serum amyloid P-component (SAMP)	1	0.4	0.39	0.33	1.30E-05	Serum Saraswat et al. (2017)
Q62740	Secreted phosphoprotein 24 (SPP24)	1	0.47	0.45	0.31	4.20E-03	
P13596	Neural cell adhesion molecule 1 (NCAM1)	1	0.4	0.22	0.28	8.10E-03	
Q5I0D5	Phospholysine phosphohistidine inorganic pyrophosphate phosphatase (LHPP)	1	0.26	0.2	0.19	1.50E-03	
P35444	Cartilage oligomeric matrix protein (COMP)	1	0.25	0.18	0.15	1.00E-06	Tissue Liao et al. (2003)
P42854	Regenerating islet-derived protein 3-gamma (REG3G)	1	0.15	0.06	0.16	2.60E-04	Tissue Yin et al. (2015)
P20759	Ig gamma-1 chain C region (IGHG1)	1	8.39	12.52	–	7.00E-07	
Q9JI85	Nucleobindin-2 (NUCB2)	1	4.14	–	–	2.50E-03	
Q63257	Interleukin-4 receptor subunit alpha (IL4RA)	1	0.5	–	–	3.10E-03	
P08932	T-kininogen 2 (KNG2)	1	–	3.32	3.84	6.00E-05	
P13265	Glypican-3 (GPC3)	1	–	0.45	0.45	2.07E-03	
P08592	Amyloid beta A4 protein (APP)	1	–	0.42	0.44	5.34E-03	
Q00657	Chondroitin sulfate proteoglycan 4 (CSPG4)	1	–	0.38	0.34	8.60E-04	Serum Keleg et al. (2014)
P01830	Thy-1 membrane glycoprotein (THY1)	1	–	0.35	0.32	2.88E-02	Tissue Zhu et al. (2014)
P04937	Fibronectin (FINC)	1	–	0.4	0.31	5.60E-04	Tissue Zhang et al. (2016)
P24090	Alpha-2-HS-glycoprotein (FETUA)	1	–	0.24	0.2	7.20E-04	Tissue, urine Schonemeier et al. (2016)

P82450	Sialate O-acetylesterase (SIAE)	1	–	0.42	–	1.13E-02	
O54861	Sortilin (SORT)	1	–	0.42	–	3.67E-02	
Q9Z1Y3	Cadherin-2 (CADH2)	1	–	0.40	–	3.04E-04	
O54715	V-type proton ATPase subunit S1 (VAS1)	1	–	0.33	–	1.77E-02	
P51635	Alcohol dehydrogenase [NADP(+)] (AKR1A1)	1	–	0.32	–	2.92E-02	
P42123	L-lactate dehydrogenase B chain (LDHB)	1	–	0.30	–	1.79E-02	
P47853	Biglycan (BGN)	1	–	0.26	–	4.00E-08	
P01041	Cystatin-B (CSTB)	1	–	0.24	–	3.09E-04	
O08628	Procollagen C-endopeptidase enhancer (PCOLCE)	1	–	0.21	–	3.17E-04	
B0BNN3	Carbonic anhydrase 1 (CA1)	1	–	–	7.72	2.26E-02	Tissue Sheng et al. (2012)
P15083	Polymeric immunoglobulin receptor (PIGR)	1	–	–	2.12	2.82E-03	Serum Tonack et al. (2013)
P10252	CD48 antigen (CD48)	1	–	–	0.49	3.80E-05	
P17164	Tissue alpha-L-fucosidase (FUCO)	1	–	–	0.49	2.26E-02	Serum, urine Dariusz Szajda et al. (2010)
Q6AYS7	Aminoacylase-1A (ACY1A)	1	–	–	0.48	2.82E-03	
P50123	Glutamyl aminopeptidase (AMPE)	1	–	–	0.47	7.29E-03	
P60711	Actin, cytoplasmic 1 (ACTB)	1	–	–	0.46	1.53E-02	Tissue Yan et al. (2016)
Q63772	Growth arrest-specific protein 6 (GAS6)	1	–	–	0.44	3.50E-05	Serum Uehara et al. (2009)
Q5XI43	Matrix-remodeling-associated protein 8 (MXRA8)	1	–	–	0.44	1.00E-04	
P18292	Prothrombin (F2)	1	–	–	0.43	3.67E-02	Serum Saraswat et al. (2017)
Q6MG61	Chloride intracellular channel protein 1 (CLIC1)	1	–	–	0.4	1.56E-02	
Q6AYT0	Quinone oxidoreductase (CRYZ)	1	–	–	0.39	1.49E-03	
P38918	Aflatoxin B1 aldehyde reductase member 3 (AKR7A3)	1	–	–	0.39	3.40E-02	

(continued)

Table 16.2 (continued)

Accession	Protein name	Fold change				*P* value	Reported to be related to pancreatic disease
		W0	W2	W3	W4		
Q64319	Neutral and basic amino acid transport protein rBAT (SLC3A1)	1	–	–	0.33	7.95E-04	
P28826	Meprin A subunit beta (MEP1B)	1	–	–	0.33	6.06E-03	
P19468	Glutamate--cysteine ligase catalytic subunit (GCLC)	1	–	–	0.26	1.21E-03	
Q64602	Kynurenine/alpha-aminoadipate aminotransferase, mitochondrial (AADAT)	1	–	–	0.22	4.00E-05	
Q9JJ40	Na(+)/H(+) exchange regulatory cofactor NHE-RF3 (PDZK1)	1	–	–	0.19	1.36E-03	
Q62687	Sodium-dependent neutral amino acid transporter B(0)AT3 (S6A18)	1	–	–	0.17	5.42E-04	

– means does not reach the criteria (fold change ≥ 2 or ≤ 0.5, $P < 0.05$) compared with week 0

cell adhesion and migration, nerve regeneration, and fibrosis, and increased expression of THY1 was observed in the activated fibroblasts (Zhu et al. 2014). FINC was expressed in the pancreatic tissue of DBTC-induced CP mice (Zhang et al. 2016). FETUA was found in the inflammatory stroma of pancreatitis. Urinary FETUA peptide was significantly differentially expressed in some patients with CP and PC (Schonemeier et al. 2016). Carbonic anhydrase 1 (CAH1) was reported to be upregulated in the pancreatic tissues of PC patients (Sheng et al. 2012). Polymeric immunoglobulin receptor (PIGR) was increased in the serum of patients with CP (Tonack et al. 2013). Tissue alpha-L-fucosidase (FUCO) is a lysosomal exoglycosidase. In PC, FUCO was shown to be a biomarker that increased in serum and decreased in urine (Dariusz Szajda et al. 2010). The expression levels of actin, cytoplasmic 1 (ACTB) were found to be upregulated significantly in the pancreatic tissue of rats with acute pancreatitis (AP) induced by caerulein (Yan et al. 2016). GAS6 regulates islet beta cells, and the plasma concentrations of GAS6 correlate with AP severity and may be a potential marker of AP (Uehara et al. 2009). Prothrombin was reported to be a candidate biomarker for differential diagnosis between CP and PC since F2 was increased in the serum of CP patients but decreased in the serum of PC patients (Saraswat et al. 2017).

Twelve differential proteins were identified before obvious pathological appearance at week 2, and these proteins were also differentially expressed at two later time points. In addition to B2M, SAMP, COMP, and REG3G, several differential proteins were involved in the regulation mechanism or development process of pancreatic disease. For example, neural cell adhesion molecule 1 (NCAM1) expression is associated with the degenerative process in pancreatic tissue with chronic inflammation (Naito et al. 2006). Secreted phosphoprotein 24 (SPP24) inhibits growth of human pancreatic cancer cells caused by the expression of BMP-2 (Li et al. 2015). Dipeptidyl peptidase 2 (DPP2) enzyme activity is essential for preventing hyperinsulinemia and maintaining glucose homeostasis (Danilova et al. 2009). Endothelial cell-selective adhesion molecule (ESAM) was reported to be associated with increased oxidative stress in type 2 diabetic patients (Kacso et al. 2014). The identified differential urinary proteins may provide important clues for the early diagnosis of CP.

16.2.3 Inflammatory Bowel Disease

Inflammatory bowel disease (IBD) is a group of chronic, relapsing intestinal inflammatory disorders with unknown etiology. The subtypes of IBD consist of Crohn's disease (CD), ulcerative colitis (UC), and IBD unclassified (IBDU) (R). According to epidemiologic data, the incidence and prevalence of IBD are increasing with time in both high-income countries and newly industrialized countries (Ng et al. 2018; Kaplan and Ng 2016), indicating that it has become a global disease. In fact, 5–15% of cases are tentatively diagnosed as IBDU since no clear assignment for CD or UC can be made, and approximately 80% of those cases can later develop into UC or

CD (Abraham et al. 2012; Melmed et al. 2007; Henriksen et al. 2006). Current diagnosis and differential diagnosis mainly rely on clinical manifestations and endoscopic, radiological, and histological criteria (Dignass et al. 2012; Tontini et al. 2015; Gomollon et al. 2017). Endoscopy and imaging have notable limitations of cost, inconvenience, and invasiveness, making these procedures unsuitable for frequent monitoring of patients with IBD. Hence, there is a need for simple, noninvasive, and accurate biomarkers for the diagnosis and prognosis of IBD diseases.

Trinitrobenzene sulfonic acid (TNBS)-induced colitis is a hapten-induced colitis model that elicits a Th1-mediated immune response that involves IL-12 and TNF-α as cytokines (Wirtz et al. 2017; Elson et al. 1996). From the immunological and histopathological characteristics, TNBS-induced colitis resembles features of CD (Kiesler et al. 2015). Male Wistar rats (180–200 g) were fasted for 24 h with water supplied. Then 10 mg TNBS dissolved in 0.25 ml of 50% ethanol was administered rectally after ether anesthesia, and rats from the control group were given the same volume of 0.9% saline according to the procedure described by Morris et al. (Morris et al. 1989). Rats were individually placed in rat fixators for 4 h to collect urine samples on days 2 and 7. The rats in the TNBS-treated group showed hypomotility and piloerection, while the feces texture presented as dilute and mucous-like and continued to increase in severity to pus and blood. The macroscopic and HE-stained microscopic features of the colon from control rats were typical and showed a normal structure: intestinal mucosa was clear and smooth (Fig. 16.3a); the epithelial cell layer was accompanied by the presence of goblet cells in straight tubular glands; and a normal quantity of cells were in the lamina propria. On day 2, macroscopic inspection showed severe mucosal damage and hemorrhage. Under the microscope, the lesions could be seen and were mainly confined to the mucosa and submucosa with neutrophil infiltration and no tissue hyperplasia. On day 7, macroscopic inspection showed prominent intestinal adhesion and colonic wall thickening, while inspection under the microscope showed diffuse inflammation extending through the muscularis mucosae, loss of the epithelium and goblet cells, and thickened lamina propria with the presence of fibroblasts (Fig. 16.3b).

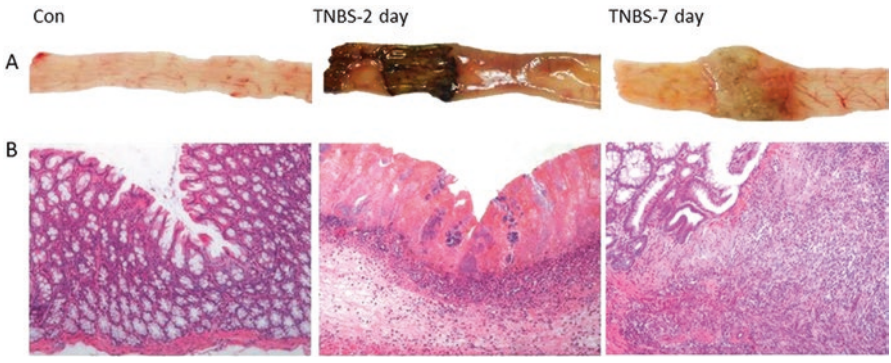

Fig. 16.3 Histopathological characterization of TNBS-induced colitis. (**a**) Macroscopic inspection; (**b**) HE staining at an original magnification 20× (Qin et al. 2019)

Urine samples from the control group, TNBS group, on day 2 and day 7 were analyzed using label-free and TMT-labeled proteomic quantitative methods. In the label-free analysis, 22 and 32 proteins with significantly differential abundance (twofold change, $p < 0.05$) were identified on day 2 and day 7, respectively. In the TMT-labeled analysis, 16 and 27 urinary proteins with significantly differential abundance (1.2-fold change, $p < 0.05$) were identified on day 2 and day 7, respectively. Among them, 77 proteins (human homologous proteins) changed in the urine of TNBS-induced colitis rats. For further validation of these differential proteins, a parallel reaction monitoring (PRM) targeted proteomic quantitative method was used to analyze other urine samples from the control group ($n = 8$), the TNBS 2-day group ($n = 11$), and the TNBS 7-day group ($n = 11$). Fifty-three proteins eventually exhibited the same average trend for the differential abundance of the proteins observed by both targeted and untargeted proteomic methods. Eighteen proteins show changes that were statistically significant (twofold change, adjust $p < 0.05$) on day 2, and 9 proteins (4 increased and 5 decreased) show changes that were statistically significant on day 7 (Table 16.3, Fig. 16.4). Among the proteins showing significant changes, five proteins changed at both time points, and the trends were consistent. From the histopathology investigations, the second day of TNBS-induced colitis displayed mainly acute intestinal mucosal injury due to the effect of ethanol in the enema. The histopathological characteristics on day 7 resemble features of human immune inflammatory bowel disease (Crohn's disease). Therefore, nine proteins showing differential abundance on day 7 have the potential to be urinary biomarkers of inflammatory bowel disease.

Among the nine PRM-validated proteins on day 7, three were highly enriched in the gastrointestinal tract based on the human protein tissue atlas (Uhlen et al. 2015).

Table 16.3 Details of the changed urinary proteins identified by PRM on day 7 (Qin et al. 2019)

Uniprot ID	Protein name	Day 2		Day 7		Human ortholog
		FC	Adjust p	FC	Adjust p	
Q8VD89	Ribonuclease pancreatic gamma type (RNS1G)	9.4	0.0013	12.4	0.0004	P07998
P30152	Neutrophil gelatinase associated lipocalin (NGAL)	29.8	0.0003	4.3	0.0429	P80188
O88766	Matrix metalloproteinase-8 (MMP-8)	2.7	0.0080	2.8	0.0052	P22894
P04797	Glyceraldehyde-3-phosphate dehydrogenase (G3P)	0.5	0.0216	0.5	0.0288	P04406
Q64319	Solute carrier family 3 member 1 (SLC31)	0.5	0.0362	0.4	0.0026	Q07837
Q62687	Solute carrier family 6 member 18 (S6A18)	0.6	0.1231	0.3	0.0004	Q96N87
Q4FZV0	Beta-mannosidase (MANBA)	1.4	0.0763	1.8	0.0328	O00462
Q9ESG3	Collectrin (TMM27)	0.7	0.4509	0.4	0.0262	Q9HBJ8
B0BNN3	Carbonic anhydrase 1 (CAH1)	1.0	0.9723	0.2	0.0026	P00915

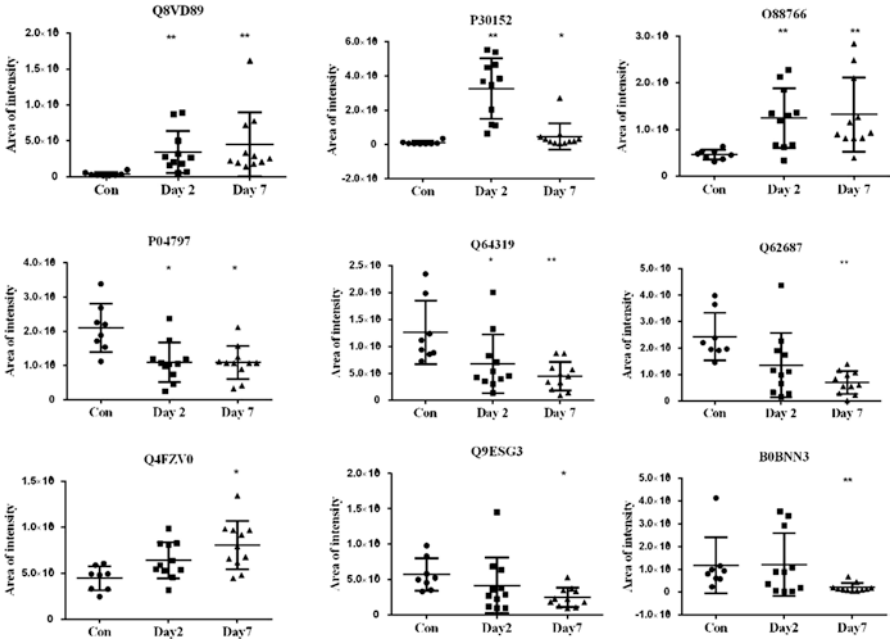

Fig. 16.4 The intensity of significantly changed urinary proteins validated by PRM on day 7. ($*p < 0.05$, $**p < 0.01$) (Qin et al. 2019)

These three proteins are ribonuclease pancreatic gamma type (RNS1G), solute carrier family 3 member 1 (SLC31), and carbonic anhydrase 1(CAH1). Additionally, three proteins had been reported as biomarkers of inflammatory bowel disease. Neutrophil gelatinase-associated lipocalin (NGAL) has been shown to be significantly increased in the urine of Crohn's disease patients compared to baseline samples (Bolignano et al. 2010), while it showed neither significant difference among patients and controls nor correlation with disease activity in another study (Oikonomou et al. 2011). It is also found to be upregulated in the serum and fecal samples of CD patients (Nielsen et al. 1999). Matrix metalloproteinase-8 (MMP-8) is reported to be significantly elevated in plasma in patients with inflammatory bowel disease compared to that in normal controls (Makitalo et al. 2012). Carbonic anhydrase 1(CAH1) is reported to be observed at lower levels in the urine of patients with Crohn's disease and ulcerative colitis compared to that found in a normal control group (Han et al. 2013). There were also some differential proteins discovered that have never been reported to be related to inflammatory bowel disease, such as glyceraldehyde-3-phosphate dehydrogenase (G3P), solute carrier family 6 member 18 (S6A18), beta-mannosidase (MANBA), and collectrin (TMM27), as well as RNS1G and SLC31. Since these proteins were dramatically changed, they also have the potential to be urinary biomarkers of inflammatory bowel disease.

16.3 Conclusions

As the lack of urinary proteomics studies in digestive diseases, these researches have provided valuable clues of urine biomarkers for diagnosis and prognosis in various digestive diseases. Disease-associated differential proteins were identified in each rat model. A greater number of urine changes occurred in the TAA-induced liver fibrosis model even in the early stage at week 1, which was earlier than the appearance of the changes in ALT and AST in the serum and fibrosis in the liver. Changes in urinary proteins can be identified before the appearance of obvious pathological changes in DDC-induced CP rats. The changed urinary proteins in TNBS-induced colitis animal model were more likely to reflect the colonic histological changes: transmural inflammation and patch hypertrophy. In addition, only one kind of rat model was used to discover urinary biomarkers; further analysis of other animal models may provide more sensitive and specific candidate biomarkers. Furthermore, a large number of clinical samples should be used to verify specific proteins or protein patterns as clinically applicable biomarkers. To validate these urinary proteins in clinical samples, targeted proteomic approaches will be used in the future.

Acknowledgments This chapter is based on published articles:

1. Zhang F, Ni Y, Yuan Y, Yin W, Gao Y. Early urinary candidate biomarker discovery in a rat thioacetamide-induced liver fibrosis model. Science China Life Sciences. 2018; 61(11):1369–1381.
2. Zhang L, Li Y, Gao Y. Early changes in the urine proteome in a diethyldithiocarbamate-induced chronic pancreatitis rat model. J Proteomics. 2018;186:8–14.
3. Qin W, Li L, Wang T, Huang H, Gao Y. Urine proteome changes in a TNBS-induced colitis rat model. Proteomics Clin Appl. 2019; e1800100. https://doi.org/10.1002/prca.201800100.

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Chapter 17
Serial Changes of Urinary Proteome in Animal Models of Renal Diseases

Mindi Zhao and Yuan Yuan

Abstract Urine is directly connected to the urinary system; as a result, urine proteome was usually used for renal diseases. As urine accumulates all types of changes, identifying the precise cause of changes in the urine proteome is challenging and crucial in biomarker discovery. To reduce the confounding factors to minimal, some studies used animal model resembling human diseases. This chapter highlights the importance of animal models and introduces two strategic researches which focused on serial changes of urine proteome in animal model of focal segmental glomerulosclerosis and tubular injury. In these studies, urine samples were collected at different stages of animal models, and urinary proteins were profiled by LC-MS/MS. For the focal segmental glomerulosclerosis model, 25 urinary proteins changed in the whole process. For the unilateral ureteral obstruction model, 7 and 19 significantly changed in the 1- and 3-week groups, respectively. We think these stage-dependent dynamic changes of urine proteome in animal models will help to support the role of urine as key source in biomarker discovery especially in kidney diseases and help to identify corresponding biomarkers for clinical validation.

Keywords Urinary proteome · Renal diseases · Animal models

17.1 Using Animal Models to Study Renal Diseases

Due to the site of the formation and regulation of urine, the urine proteome has been widely investigated in studies of kidney, bladder, and prostate diseases (Klein and Schanstra 2018; Decramer et al. 2008). As urine accumulates all types of changes, identifying the precise cause of changes in the urine proteome is challenging and crucial in biomarker discovery. The advantages of using animal models are the

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following: (1) reducing the effects of genetic and environmental factors on the urine proteome as much as possible; (2) knowing the exact time of disease occurrence, which is helpful in the identification of biomarkers for each stages including early detection; and (3) avoiding the effects of medications on the urine proteome because therapeutic measures for patients are inevitable.

Using animal model to find clues and validate them in clinical samples may be a good way to discover biomarkers in urine. Some stage-specific proteomics studies have been conducted in some animal model studies, most of them using two-dimensional gel electrophoresis coupled to MALDI-TOF mass spectrometry, for example, streptozotocin-induced diabetic nephropathy in rats (Sharma and Tikoo 2014) and rat models of passive Heymann nephritis (Ngai et al. 2006). As two-dimensional gel electrophoresis is time-consuming, essentially nonquantitative and limited peptides could be identified (Haynes and Yates 2000). In this chapter, we mainly introduce an adriamycin-induced nephropathy (ADN) and a unilateral ureteral obstruction (UUO) rat model study which observed dynamic changes of urinary proteins by LC-MS/MS.

17.2 Dynamic Changes of Urine Proteome in an ADN Model

With a significantly increasing frequency in the past 20 years, focal segmental glomerulosclerosis (FSGS) is currently a common cause of nephrotic syndrome and responsible for 15% end-stage renal disease (Kitiyakara et al. 2003). Only renal biopsy can distinguish different types of nephrotic syndrome. However, FSGS is "focal" and "segmental," so the diagnosis of FSGS is often more problematic and complex (Thomas 2009). Adriamycin (ADR)-induced nephropathy is the most prototypical and commonly used experimental model of human primary FSGS (Pippin et al. 2009).

For the dynamic changes of urine proteome in the ADN model, urine samples were collected at days 0 (before injection), 3, 7, 11, 15, and 23 after the intravenous injection of ADR (Zhao et al. 2014) and analyzed by LC-MS/MS. The enrichment of ConA-binding urinary proteins was conducted to enrich the glycoprotein in the proteomic analysis, as described previously (Wang et al. 2008).

By label-free quantitative and statistical analyses, 25 proteins met the following conditions: (1) compared with day 0, max fold change>2 in each rat and (2) p value<0.05. Among the 25 changed proteins, 14 proteins shared an overall increasing trend in relative abundance, and 9 proteins shared an overall decreasing trend. Three trends were observed in these candidate biomarkers during ADR-induced nephropathy progression. The first was a gradual increase, with examples including afamin and ceruloplasmin. The second was a gradual decrease, with examples including cadherin-2 and aggrecan core protein. The third, which includes fetuin-B and beta-2-microglobulin, was early changes with distinct patterns. At the early stage, such as 3 and 7 days after the ADR injection, proteinuria was not obvious.

However, several proteins, such as fetuin-B, AMBP, and kallikrein-1, were decreased during this phase. These proteins may be good candidates for the early detection.

A previous study compared the kidney input (plasma) and output (urine) proteomes and divided urinary proteins into three categories, the plasma-only subproteome, the plasma-and-urine subproteome, and the urine-only subproteome (Jia et al. 2009). To further analyze the functions of these candidate biomarkers, these changed proteins were compared with the human plasma proteome (Saha et al. 2008), human urine proteome (Marimuthu et al. 2011; Li et al. 2010; Adachi et al. 2006), and kidney origin proteome (Jia et al. 2013). Most changed proteins exist in the normal human plasma proteome (18/21) and urine proteome (17/21); however, the CD166 antigen was detected only in the plasma proteome, kallikrein-1 was detected only in the normal urine proteome, and 10 proteins were detected in the kidney origin proteome.

Candidate biomarkers identified by progressive ADR-induced nephropathy were compared with a manually curated human and animal urinary protein biomarker database (Shao et al. 2011). Some proteins were found to be candidate biomarkers for other glomerular diseases; for example, elevated afamin expression has been reported in some diabetic nephropathies. The similarities may due to a common pathway of different glomerular diseases at later stages (Kriz et al. 1998).

17.3 Serial Changes of Urine Proteome in a UUO Model

In obstructive nephropathy, interstitial fibrosis is caused by tubular injury and the proliferation of interstitial fibroblasts, and this process is considered the most serious pathological change in end-stage renal disease (Chevalier et al. 2009; Rampanelli et al. 2014). Few studies have focused on identifying urinary biomarkers involved in this pathological process (Tampe and Zeisberg 2014). The UUO model mimics different stages of obstructive nephropathy and possibly leads to the infiltration of inflammatory cells, tubular expansion and apoptosis, myofibroblast accumulation, and differentiation.

For the dynamic changes of urine proteome in the UUO model, 1- and 3-week urine samples were collected from the residuary ureter linked to the kidney following UUO and analyzed by LC-MS/MS (Yuan et al. 2015). Relative to the sham-operated group, 65 differentially expressed proteins were found in the UUO 1-week group and 74 in the UUO 3-week group. Fifty-one overlaps existed between these two sets of differential proteins. Overall, 7 significantly changed proteins were observed between the sham-operated and UUO 1-week groups and 19 significantly changed proteins between the sham-operated and UUO 3-week groups. Two overlaps were observed between these sets of differentially expressed proteins.

Levels of several differential proteins identified between the sham-operated group and the UUO group, such as aminopeptidase N, vimentin, and lumican, had previously been reported to be higher in damaged kidney tissue in obstructive

nephropathy studies (Schaffer et al. 2002; Silverstein et al. 2003; Grande and Lopez-Novoa 2009). Although several of the proteins identified were not related to obstructive nephropathy, they were associated with other renal diseases and served as potential markers of various pathologies. For example, the significant upregulation of glycogen phosphorylase has been reported in diabetic nephropathies (Kandasamy and Ashokkumar 2014). Cathepsin D promotes the fibrogenic potential of hepatic stellate cells (Moles et al. 2009) and is increased in the saliva of cystic fibrosis patients (Minarowska et al. 2007). Most of the differentially expressed proteins had not been detected in urine from the UUO model. In this study, the differential proteins in urine were considered to play an important role in the regulation of obstructive nephropathy and possibly serve as key markers during the progression of this disease. The proteins that changed after 1 week of UUO may serve as candidate renal tubular injury biomarkers, whereas proteins changed after 3 weeks of UUO may reflect renal interstitial fibrosis.

17.4 Comparison of Urine Proteome Changes in Two Renal Diseases

Several differentially expressed proteins appear in many different nephropathies, which suggest that these proteins may be the common markers of multiple diseases that share similar pathological changes. Distinct differential proteins were present in the ADR-induced nephropathy and UUO models. Serum albumin, serotransferrin, and Kallikrein-1 were detected in both studies but displayed opposite trends. In the UUO 1-week group, the glomerular morphology was almost normal, whereas obvious glomerular injury appeared in the UUO 3-week group. Kallikrein-1 decreased 3 weeks after UUO and in the ADR-induced nephropathy model, which indicates that Kallikrein-1 is a candidate marker of glomerular injury. The expression profile of differentially expressed proteins in these studies illustrates that specific pathological conditions have their own characteristic changes and biomarker combinations.

17.5 Future Perspective

The advantage of studying urine proteome by animal models is that urine proteome is subjected to many factors like diet, drugs, and lifestyles; increasing the number of clinical samples is laborious and almost futile with current sample analysis throughput. We think finding clues from well-controlled animal experiment and validating them in clinical samples are better ways to develop biomarkers in urine. These changed urinary protein panel should be validated in clinic in the future. As the same urinary proteins appeared to be modulated in several renal diseases, for

example, albumin was increased in both diabetic nephropathy and IgA nephropathy. Thus, it may be difficult to provide an accurate diagnosis using a single biomarker; a panel of urinary proteins may be more specific and more sensitive. We hope this strategy will help to support the role of urine as key source in biomarker discovery especially in kidney diseases and help to identify biomarkers for clinical validation.

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Part III
Factors that Interfere Urinary Biomarkers

Chapter 18
Effects of Extrinsic Factors on the Urinary Proteome

Menglin Li

Abstract As an early and sensitive source of biomarker library, urine is attracting increasing attention from researchers in different fields. The urinary proteome is being explored for their diagnostic capabilities, ability to monitor disease and prognostic utility. However, the urine component is extremely complicated mainly due to the disturbance introduced by various conditions, including intrinsic and extrinsic factors. Intrinsic factors including gender, age, and daily rhythms are usually appreciated in clinical biomarker studies, but external factors are rarely taken into consideration. In this review, the effects of common extrinsic factors such as drugs, diet, physical activity, and extreme environments on human urine are summarized and discussed. These factors and the corresponding influence should be emphasized when biomarker studies are conducted. It will facilitate us to avoid costly phony candidates.

Keywords Biomarker · Extrinsic factors · Proteomics · Urine

18.1 Introduction

The intrinsic properties of urine make it a gold mine for biomarker discovery in clinical proteomic studies (Gao 2013). Information originated from proximal tissues and blood perfusing distant organs can be retained in urine. In addition to its application in urogenital diseases, urine has been applied to a wide range of nonurogenital diseases to exploit their potential biomarkers (Pedroza-Díaz and Röthlisberger 2015).

On the other hand, because urine can enrich changes from different systems of the body, this characteristic makes it a highly sensitive matrix that can indicate physiological and pathophysiological changes in the body. This sensitivity can impact the screening and subsequent validation of candidate disease biomarkers. Various physiological factors can affect the urine proteome, such as gender, age,

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menstrual cycle, and hormone status. More importantly, the abundance of urinary candidate biomarkers could also be affected by various conditions (Wu and Gao 2015; Huan et al. 2018). In addition to the intrinsic factors, extrinsic factors also exhibit great impact on urine protein component. Extrinsic factors are stimuli from external environments. The body will undergo adaptive changes after stimulation, and changes will be likely reflected in blood and urine. Besides, some factors cannot be avoided and thus are difficult to be balanced in experiment design. For example, taking medicine is indispensable for patients; alteration in abundance in urinary biomarkers is the result of the compromise between disease and drugs because both of them can introduce changes (Gao 2014a). Therefore, figuring out the influence of extrinsic factors upon urine proteome is necessary and urgent. In this review, we summarize the impact of different extrinsic factors on urinary proteome and list the proteins that these factors could affect. We wish that researchers could obtain reliable biomarkers by taking advantage of the protein information provided.

18.2 Drugs

In clinical practice, medicine can control disease processes and possibly introduce a significant impact on the urinary proteome. Thus, the alteration of urinary proteins can be from either disease or medicine or even both. Besides, therapeutic effects cure the illness and might abolish disease biomarkers. If the associations of disease-medication and healthy control-no medication were not extensively interpreted, it would hinder researchers to differentiate the source of biomarkers (diseases associated or medicine associated) (Gao 2014a). However, when clinical biomarker studies were designed, factors such as gender, age, disease progression, and complications were always emphasized, and the medicines taken by the patients were usually ignored. This situation will reduce the effectiveness and applicability of the resulting biomarkers. The necessity of ruling out the effects of various drugs on biomarkers should be emphasized (Gao 2014b). To extensively understand the potential influence of drug usage on urinary proteins, we summarized the researches of different drugs on urinary proteome.

18.2.1 Glucocorticoid

Glucocorticoids are widely used to treat various diseases based on their potent anti-inflammatory and immunosuppressive effects. It was estimated that 1.2% of the US population used oral glucocorticoids continuously for more than 5 years (Overman et al. 2013). Prednisone is a commonly prescribed glucocorticoid that has a strong association with protein metabolism. Based on the rat model, using LC-MS/MS analysis, Wu et al. identified 27 urinary proteins showing significant difference after prednisone treatment (Wu et al. 2017). Specifically, 12 proteins were increased, and

15 proteins were decreased in abundance. A total of 16 proteins and their human orthologs have been previously annotated as disease biomarkers, suggesting the potential impact of prednisone treatment on urine biomarkers. Notably, the expression of seven differential proteins after prednisone treatment was similar to levels in specific disease conditions, including alpha-1-acid glycoprotein, serine protease inhibitor A3N, haptoglobin, alpha-enolase, urinary protein 1, urinary protein 2, and nuclear pore complex protein Nup107. These proteins might be erroneously considered as disease biomarkers if the drug effect was not taken into account. Therefore, attention should be paid to the effects of prednisone treatment in future disease biomarker studies.

18.2.2 Anticoagulants and Procoagulants

Aspirin is widely used to reduce the risk of myocardial infarction (MI) and cardiovascular disease (CVD) deaths, as well as reduce cancer risk, cancer metastasis, and cancer mortality (Liao et al. 2012). In 2010, 19.0% of US adults reported taking aspirin regularly for at least 3 months (Zhou et al. 2014). Accordingly, a better understanding of its effects on the urinary proteome is necessary for advancing urinary biomarker research. Li et al. examined the effects of low-dose aspirin (50 mg/kg/day for 7 days) on the urinary proteome using label-free quantitative proteomics based on a rat model. Twelve proteins were significantly altered after aspirin administration, and 9 of the 12 were reported as candidate disease biomarkers (pro-epidermal growth factor, CD10, meprin A subunit alpha, uromodulin, actin, glyceraldehyde-3-phosphate dehydrogenase, dipeptidyl peptidase 4, beta-2-microglobulin, pro-epidermal growth factor), suggesting that these effects must be considered in future urinary biomarker research.

Heparin is another worldwide used anticoagulant medicine. Apart from its anticoagulant application, it can also reduce the adverse ischemic outcomes, including death and myocardial infarction (Cohen et al. 1994), and exhibit potency of anti-inflammatory and anti-tumor (Oduah et al. 2016). Argatroban, a small molecule that selectively inhibits thrombin, is more effective than heparin in preventing arterial thrombosis (Yeh and Jang 2006). Li et al. analyzed the changes in urinary proteome after two different anticoagulants were administrated to rats (heparin or argatroban) (Li et al. 2014a). 62/27 proteins have shown significant alteration in abundance after heparin/argatroban treatment, respectively. Among these changed proteins, ten proteins were annotated as candidate disease biomarkers in the heparin-treated group, including complement C4, afamin, serum albumin, beta-2-glycoprotein 1, pro-epidermal growth factor, complement C3, deoxyribonuclease-1, serine protease inhibitor A3N, kallikrein-1, and plasminogen. In argatroban group, 20 proteins were candidate biomarkers, such as alpha-1-antiproteinase, C-reactive protein, fibronectin, neprilysin, glutathione S-transferase P, elongation factor 1-alpha 1, T-kininogen 2, serine protease inhibitor A3M, beta-2-glycoprotein 1, serine protease inhibitor A3N, plasminogen, complement C4, vitamin D-binding protein, Ig gamma-1 chain

C region, complement C3, afamin, ceruloplasmin, serum albumin, neutral and basic amino acid transport protein rBAT, and meprin A subunit beta. Interestingly, only one protein (afamin) was affected by all of the three anticoagulants (aspirin, heparin, and argatroban). This reminds us that the urinary proteome can distinguish the changes in blood coagulation status introduced by different drugs.

On the other hand, vascular disruption with concomitant hemorrhage is a leading cause of death in civilian and military trauma (Blackbourne et al. 2010). Procoagulants including ε-aminocaproic acid (EACA), p-aminomethylbenzoic acid (PAMBA), and tranexamic acid (TXA) are widely used in combat injury and reduced mortality (Morrison et al. 2012). Currently, there are still no biomarkers for predicting its effect on biofluids. Jing et al. analyzed two different procoagulants (EACA, TXA) by LC-MS/MS (Jing et al. 2018, 2019;) on rat models. After TXA treatment, 28 differential urinary proteins were detected, of which 13 had been previously considered as candidate biomarkers (Jing et al. 2018), including selenium-binding protein 1, apolipoprotein M, myoglobin, urinary protein 1, prosaposin (sulfated glycoprotein 1), superoxide dismutase [Cu-Zn], ephrin-B1, nidogen-2, interleukin-4 receptor subunit alpha, fumarylacetoacetase, calbindin, guanine nucleotide-binding protein G (I)/G(S)/G(T) subunit beta-1, and WAP four-disulfide core domain protein 2. After EACA treatment, 65 proteins have shown significant changes in urine; 22 of these proteins are potential biomarkers for various disease (Jing et al. 2019). These proteins are Rho GDP-dissociation inhibitor 1, stanniocalcin-1, cathepsin S, alkaline phosphatase, cystatin-related protein 2, anthrax toxin receptor 1, selenium-binding protein 1, beta-2-microglobulin, complement C3, glutathione S-transferase P, chondroitin sulfate proteoglycan 4, podocalyxin, serum albumin, sulfhydryl oxidase 1, CD59 glycoprotein, prostasin, thioredoxin, fumarylacetoacetase, Na(+)/H(+) exchange regulatory cofactor NHE-RF3, neutrophil gelatinase-associated lipocalin, clusterin, and ceruloplasmin. In particular, the shared differential urinary proteins they identified between EACA- and TXA-treated groups are rare, and only four differential proteins are included, such as fumarylacetoacetase, frizzled-2, D-dopachrome decarboxylase, and selenium-binding protein 1.

18.2.3 Diuretics

Diuretics are among the most commonly used medications. They are used to induce negative fluid and sodium balances in a variety of clinical situations, including congestive cardiac failure and hypertension, nephritic syndrome, and cirrhosis (Wile 2012). However, it remains unclear whether and how diuretics affect the urinary proteome. Li et al. examined the effects of three types of diuretics (furosemide, hydrochlorothiazide, and spirolactone) on the urinary proteome using label-free quantitative proteomics (Li et al. 2014b). These drugs represent thiazide diuretics, loop diuretics, and potassium-sparing diuretics, respectively. Fourteen proteins showed significant difference after diuretics were applied. There are seven

differential proteins for furosemide-treated group, five for spirolactone, and two for hydrochlorothiazide. Five of the seven proteins in the furosemide group and five proteins in the spirolactone group have been reported as candidate disease biomarkers. These proteins are submandibular glandular kallikrein-9, secretoglobin family 2A member 2, cystatin-related protein 2, osteopontin, plasminogen, haptoglobin, urinary protein 2, urinary protein 1, sulfated glycoprotein 1, and trefoil factor 2. Interestingly, no significantly changed proteins are shared by any two groups, indicating the distinct effects of the diuretics on the urinary proteome. The results obtained here could help minimize the interference of diuretics with biomarker discovery using the urinary proteomics.

18.2.4 Anesthetics

In animal experiments, anesthetics are commonly used to investigate disease biomarkers. It is necessary to figure out whether anesthesia affects the urine proteome and interferes biomarker studying. Zhao et al. analyzed the effects of two anesthetics (pentobarbital sodium and chloral hydrate) on the rat urine proteome using LC-MS/MS. The relative abundance of 22/23 urinary proteins was changed with pentobarbital sodium or chloral hydrate anesthesia, respectively. In the pentobarbital sodium anesthesia group, 11 out of 22 proteins were considered as candidate biomarkers, including alpha-1-antiproteinase, cathepsin L1, pro-epidermal growth factor, uromodulin, serum albumin, serotransferrin, gamma-glutamyl hydrolase, aminopeptidase N, glandular kallikrein-7, submandibular/renal, meprin A subunit alpha, and neutral and basic amino acid transport protein rBAT. Among these proteins, some exhibited the opposite trend. For example, the relative abundance of aminopeptidase N was increased in septic rats with acute renal failure (Wang et al. 2008), whereas their relative abundance decreased with pentobarbital sodium anesthesia. So usage of pentobarbital sodium may underestimate the importance of this protein biomarker. In the chloral hydrate anesthesia group, 8 out of 23 differential proteins were considered as candidate biomarkers, including alpha-1-antiproteinase, cathepsin L1, pro-epidermal growth factor, uromodulin, parvalbumin alpha, superoxide dismutase [Cu-Zn], cadherin-1, and Na(+)/H(+) exchange regulatory cofactor NHE-RF3. In future biomarker discovery studies, interferences from anesthetics should be excluded.

18.2.5 α1-Adrenergic Receptor Antagonist

Changes in the central nervous system can be reflected in urine as mentioned above (Zhao et al. 2015). As the sympathetic nervous system is a critical component in the autonomic nervous system, the impact of drugs mediating sympathetic nervous system activity on urine proteome should also be revealed. Prazosin, a specific

α1-adrenergic receptor antagonist, has been used to treat cardiovascular diseases (Brogden et al. 1977) and currently is a potential drug for treating post-traumatic stress disorder (PTSD) (Berardis et al. 2015). Zhao et al. evaluated the possible impact of α1-adrenergic receptor prazosin on rat urinary proteome using TMT protein labeling, fractionation, and LC-MS/MS method (Zhao et al. 2016). A total of 775 proteins were identified, with 21 proteins significantly changed by prazosin administration (11 increased and 10 decreased). Eight significantly changed proteins were previously annotated as urinary candidate biomarkers, including major urinary protein, annex in A5, beta-2-microglobulin, carbonic anhydrase 1, lithostathine, haptoglobin, angiotensinogen, and neutral and basic amino acid transport protein rBAT. These protein changes should be considered when studying related diseases.

18.2.6 Arginine Vasopressin

Arginine vasopressin (AVP), also called antidiuretic hormone, has two most important physiological functions which are as a vasoconstrictor and an antidiuretic (Hanna and Scanlon 1997). AVP is used for the treatment of many diseases, such as diabetes insipidus, portal venous hypertension, bleeding disorders, septic shock, and cardiopulmonary resuscitation (Treschan and Peters 2006). An et al. analyzed urinary proteome after 1 week of AVP infusion (10 ng/h and 50 ng/h), eight and ten proteins had significantly altered expression in the low- and high-dose groups, respectively. Nine differential proteins are known disease biomarkers, including haptoglobin, pro-cathepsin H, regenerating islet-derived protein 3-gamma, osteopontin, calbindin, cluster of glyceraldehyde-3-phosphate dehydrogenase, CD166 antigen, complement C3, and beta-2-glycoprotein 1. We should consider the effects of AVP on urinary proteins in future urinary biomarker researches.

18.3 Summary of This Section

As reviewed in this section, a great many drugs could disturb urinary proteome and affect candidate biomarkers for various diseases. These diseases are related with different systems, including urological system (furosemide, hydrochlorothiazide, spirolactone, and arginine vasopressin), cardiovascular system (prazosin and aspirin), immunity system (prednisone), coagulation system (aspirin, heparin, argatroban, ε-aminocaproic acid, and tranexamic acid), and nervous system (pentobarbital sodium and chloral hydrate). When researches are designed to study the biomarkers or mechanisms of the corresponding diseases, the impact of the drug should be taken into consideration. The proteins that can be affected by these drugs and their relationship with specific diseases or animal models are listed in Table 18.1. *In total, 211*

Table 18.1 Details of the identified urinary proteins altered by different drug treatment

UniProt	Fold change	Description	Candidate biomarker	Related diseases
Prednisone				
P02764	1.77	Alpha-1-acid glycoprotein	Yes	↑in T1DM, DN, renal AR, AKI, acute appendicitis, vasculitis, and preeclampsia
P05545	1.61	Serine protease inhibitor A3K		↑in T2DM, renal AR, non-small cell lung carcinoma, and acute appendicitis
P09006	2.42	Serine protease inhibitor A3N	Yes	↑in T2DM, renal AR, non-small cell lung carcinoma, and acute appendicitis
P02780	2.66	Secretoglobin family 2A member 2		↓in nephrotic syndrome
P06866	2.08	Haptoglobin	Yes	↑bladder transitional cell carcinoma, AKI, DN, and T2DM
P36374	2.01	Prostatic glandular kallikrein-6		↓in acute renal failure
Q9EQV6	1.9	Tripeptidyl-peptidase 1		
O88766	1.69	Neutrophil collagenase(MMP8)	Yes	Periodontitis
P02782	2.79	Prostatic steroid-binding protein C1		
O54715	1.85	V-type proton ATPase subunit S1		
Q9R0J8	1.51	Legumain		
P84039	2.11	Ectonucleotide pyrophosphatase/ phosphodiesterase family member 5		
P23680	−1.68	Serum amyloid P-component	Yes	↑in intestinal mucosal injury
P04764	−1.57	Alpha-enolase	Yes	↓in Dent's disease
P81828	−1.61	Urinary protein 2		↓in nephrotic syndrome
P81827	−1.68	Urinary protein 1		↓in nephrotic syndrome and glomerulonephritis
P35444	−1.62	Cartilage oligomeric matrixprotein	Yes	↑in osteoarthritis
P01830	−1.9	Thy-1 membrane glycoprotein	Yes	↑in prostate cancer
O35112	−1.51	CD166 antigen	Yes	↑in T1DM
P52590	−1.99	Nuclear pore complex protein Nup107		↓in IgA nephropathy
P15473	−1.53	Insulin-like growth factor-binding protein 3	Yes	↑prostate cancer
P11030	−1.56	Acyl-CoA-binding protein		↑in Dent's disease

(continued)

Table 18.1 (continued)

UniProt	Fold change	Description	Candidate biomarker	Related diseases
P97603	−1.5	Neogenin (fragment)		
P80202	−1.89	Activin receptor type-1B		
P08649	−1.66	Complement C4		
P16310	−1.58	Growth hormone receptor		
Q5FVR0	−1.51	T-cell immunoglobulin and mucin domain-containing protein 2		
P14046	−1.71	Alpha-1-inhibitor 3		
Q01205	−1.5	2-oxoglutarate dehydrogenase complex component E2		
Aspirin				
P07522	Up	Pro-epidermal growth factor	Yes	Null
P07861	Up	CD10	Yes	Null
Q64230	Up	Meprin A subunit alpha	Yes	Null
P27590	Down	Uromodulin	Yes	Null
P60711	Down	Actin, cytoplasmic 1	Yes	Null
P04406	Down	Glyceraldehyde-3 -phosphate dehydrogenase	Yes	Null
P14740	Down	Dipeptidyl peptidase 4	Yes	Null
P07151	Up	Beta-2-microglobulin	Yes	Null
P07522	Down	Pro-epidermal growth factor	Yes	Null
P15083	Up	Polymeric immunoglobulin receptor		
Q6P9V9	Down	Tubulin alpha-1B chain		
P36953	Down	Afamin		
Q6IRK9	Up	Carboxypeptidase Q		
Heparin				
P08649	2.8	Complement C4	Yes	Null
P36953	3.7	Afamin	Yes	Null
P02770	3.1	Serum albumin	Yes	Null
P26644	2.4	Beta-2-glycoprotein 1	Yes	Null
P07522	−2.3	Pro-epidermal growth factor	Yes	Null
P01026	2.7	Complement C3	Yes	Null
P21704	−2.3	Deoxyribonuclease-1	Yes	Null
P09006	2.3	Serine protease inhibitor A3N	Yes	Null
P00758	−2.1	Kallikrein-1	Yes	Null
Q01177	3.7	Plasminogen	Yes	Null
Q63416	2.5	Inter-alpha-trypsin inhibitor heavy chain H3		
P20762	3.1	Ig gamma-2C chain C region		
P14480	2.1	Fibrinogen beta chain		
P20059	2.6	Hemopexin		

(continued)

Table 18.1 (continued)

UniProt	Fold change	Description	Candidate biomarker	Related diseases
P02680	3.4	Fibrinogen gamma chain		
P04639	3.4	Apolipoprotein A-I		
Q9QX79	2.9	Fetuin-B		
Q9R0T4	−1.5	Cadherin-1		
P24090	2.9	Alpha-2-HS-glycoprotein		
P06399	2.4	Fibrinogen alpha chain		
P12346	2.5	Transferrin		
P29598	−3.5	Urokinase-type plasminogen activator		
Q63257	−1.8	Interleukin-4 receptor subunit alpha		
P36373	−2	Glandular kallikrein-7		
P02651	3.3	Apolipoprotein A-IV		
Q64230	−5.1	Meprin A subunit alpha		
P85971	−2.5	6-Phosphogluconolactonase		
Argatroban				
P17475	1.9	Alpha-1-antiproteinase	Yes	Null
P48199	1.9	C-reactive protein	Yes	Null
P04937	1.6	Fibronectin	Yes	Null
P07861	−8.8	Neprilysin	Yes	Null
P04906	−3.6	Glutathione S-transferase P	Yes	Null
P62630	−3.4	Elongation factor 1-alpha 1	Yes	Null
P08932	2	T-kininogen 2	Yes	Null
Q63556	2.2	Serine protease inhibitor A3M (fragment)	Yes	Null
P26644	2.1	Beta-2-glycoprotein 1	Yes	Null
P09006	1.7	Serine protease inhibitor A3N	Yes	Null
Q01177	1.9	Plasminogen	Yes	Null
P08649	1.9	Complement C4	Yes	Null
P04276	1.7	Vitamin D-binding protein	Yes	Null
P20759	1.7	Ig gamma-1 chain C region	Yes	Null
P01026	1.8	Complement C3	Yes	Null
P36953	1.9	Afamin	Yes	Null
P13635	1.7	Ceruloplasmin	Yes	Null
P02770	1.5	Serum albumin	Yes	Null
Q64319	−2.3	Neutral and basic amino acid transport protein Rbat	Yes	Null
P28826	−5	Meprin A subunit beta	Yes	Null
P04639	2.3	Apolipoprotein A-I		
P29598	−6.9	Urokinase-type plasminogen activator		
P04764	−5.1	Alpha-enolase		

(continued)

Table 18.1 (continued)

UniProt	Fold change	Description	Candidate biomarker	Related diseases
Q10758	−3.1	Keratin, type II cytoskeletal 8		
Q62812	−3.7	Myosin-9		
P10111	−2.8	Peptidyl-prolyl cis-trans isomerase A		
P07314	−3.5	Gamma-glutamyltranspeptidase 1		
P36373	−2.4	Glandular kallikrein-7, submandibular/renal		
P02651	2.7	Apolipoprotein A-IV		
Q9WUW3	2.3	Complement factor I		
P11980	−2.6	Pyruvate kinase PKM		
P14740	−2.9	Dipeptidyl peptidase 4		
P04797	−3.2	Glyceraldehyde-3-phosphate dehydrogenase		
P85971	−2.3	6-Phosphogluconolactonase		
P10959	2.2	Carboxylesterase 1C		
Q99PS8	2.3	Histidine-rich glycoprotein		
Q6P734	2.1	Plasma protease C1 inhibitor		
P24090	2.3	Alpha-2-HS-glycoprotein		
P61972	−2.9	Nuclear transport factor 2		
Q9QX79	2.1	Fetuin-B		
P01048	1.8	T-kininogen 1		
Q63678	1.7	Zinc-alpha-2-glycoprotein		
P20760	1.9	Ig gamma-2A chain C region		
Q9EPH1	1.9	Alpha-1B-glycoprotein		
O70535	1.7	Leukemia inhibitory factor receptor		
Q63416	1.7	Inter-alpha-trypsin inhibitor heavy chain H3		
P20762	1.9	Ig gamma-2C chain C region		
D3ZTE0	1.7	Coagulation factor XII		
P20059	1.6	Hemopexin		
P14272	1.8	Plasma kallikrein		
P14046	2	Alpha-1-inhibitor 3		
P20761	1.8	Ig gamma-2B chain C region		
P01015	1.5	Angiotensinogen		
P12346	1.6	Ttransferrin		
P02680	2	Fibrinogen gamma chain		
Q03626	1.9	Murinoglobulin-1		
P55159	1.6	Serum paraoxonase/ arylesterase 1		
P23764	1.7	Glutathione peroxidase 3		

(continued)

Table 18.1 (continued)

UniProt	Fold change	Description	Candidate biomarker	Related diseases
Q64230	−3.7	Meprin A subunit alpha		
P14480	1.8	Fibrinogen beta chain		
P06399	2.2	Fibrinogen alpha chain		
Q6DGG1	−3.4	Alpha/beta hydrolase domain-containing protein		
Tranexamic acid				
Q8VIF7	−4.86	Selenium-binding protein 1	Yes	Acute kidney injury
P14630	−2.96	Apolipoprotein M	Yes	Acute renal injury
Q9QZ76	−3.08	Myoglobin	Yes	Damage of myocardial cells, cardiovascular disease, acute myocardial infarction
P81827	1.53	Urinary protein 1	Yes	Focal segmental glomerulosclerosis (FSGS) and mesangioproliferative glomerulonephritis (MsPGN)
P10960	−1.81	Prosaposin (sulfated glycoprotein 1)	Yes	FSGS and MsPGN
P07632	−1.69	Superoxide dismutase [Cu-Zn]	Yes	FSGS and MsPGN, immune-mediated nephritis
P52796	−4.2	Ephrin-B1	Yes	Hepatocellular carcinoma
B5DFC9	1.59	Nidogen-2	Yes	Ovarian cancer
Q63257	1.61	Interleukin-4 receptor subunit alpha	Yes	Pathologic grade and clinical stage of bladder cancer
P25093	−2.07	Fumarylacetoacetase	Yes	Protein excretion with sodium loading
P07171	−1.87	Calbindin	Yes	Protein excretion with sodium loading
P54311	4.89	Guanine nucleotide-binding protein G (I)/G(S)/G(T) subunit beta-1	Yes	Protein excretion with sodium loading
Q8CHN3	−1.54	WAP four-disulfide core domain protein 2	Yes	Tubulointerstitial fibrosis and tubular cell damage in chronic kidney disease, proliferation of human ovarian cancer cells
Q68FS4	10.33	Cytosol aminopeptidase		
P13432	6.95	SMR1 protein		
Q6P6S9	6.86	Ectonucleoside triphosphate diphosphohydrolase-5		
B0BNA5	4.4	Coactosin-like protein		
P08753	4	Guanine nucleotide-binding protein G (k) subunit alpha		
Q5FVR3	3.55	CD302 antigen		

(continued)

Table 18.1 (continued)

UniProt	Fold change	Description	Candidate biomarker	Related diseases
P54313	2.46	Guanine nucleotide-binding protein G (I)/G(S)/G(T) subunit beta-2		
Q9ESG3	2.3	Collectrin		
Q63467	−1.52	Trefoil factor 1		
P80254	−1.73	D-dopachrome decarboxylase		
P15943	−1.76	Amyloid-like protein 2		
Q64119	−2.5	Myosin light polypeptide 6		
Q08464	−2.53	Frizzled-2		
P10758	−4.89	Lithostathine		
P62161	−12	Calmodulin		
ε-Aminocaproic acid				
Q5XI73	−5.49	Rho GDP-dissociation inhibitor 1	Yes	Abdominal aortic aneurysm
P97574	−9.00	Stanniocalcin-1	Yes	Alzheimer's disease, glioma
Q02765	2.58	Cathepsin S	Yes	Atherosclerosis, abdominal aortic aneurysm
P08289	−4.20	Alkaline phosphatase	Yes	Cardiac function in pediatric patients
P22283	−2.47	Cystatin-related protein 2	Yes	FSGS and MsPGN
Q0PMD2	−2.47	Anthrax toxin receptor 1	Yes	Head and neck squamous cell carcinoma
Q8VIF7	−17.00	Selenium-binding protein 1	Yes	Heavy metal-induced nephrotoxicity
P07151	1.64	Beta-2-microglobulin	Yes	Hepatic fibrosis, nephrotoxicity
P01026	−2.00	Complement C3	Yes	Insulin resistance after bariatric surgery
P04906	−3.75	Glutathione S-transferase P	Yes	Kidney toxicity
Q00657	−19.66	Chondroitin sulfate proteoglycan 4	Yes	Melanoma
Q9WTQ2	−6.99	Podocalyxin	Yes	Membranous nephropathy
P02770	1.98	Serum albumin	Yes	Membranous nephropathy, sepsis-induced acute renal failure, FSGS andMsPGN
Q6IUU3	−2.86	Sulfhydryl oxidase 1	Yes	Neuroblastoma
P27274	2.1	CD59 glycoprotein	Yes	Obstructive chronic lung allograft dysfunction after lung transplantation
Q9ES87	−5.00	Prostasin	Yes	Ovarian cancer
P11232	2.17	Thioredoxin	Yes	Post-injury sepsis
P25093	−12.50	Fumarylacetoacetase	Yes	Protein excretion with sodium loading

(continued)

Table 18.1 (continued)

UniProt	Fold change	Description	Candidate biomarker	Related diseases
Q9JJ40	−28.6	Na(+)/H(+) exchange regulatory cofactor NHE-RF3	Yes	Protein excretion with sodium loading
P30152	1.54	Neutrophil gelatinase-associated lipocalin	Yes	Renal injury, kidney toxicity
P05371	−3.64	Clusterin	Yes	Renal injury, kidney toxicity
P13635	2.47	Ceruloplasmin	Yes	Uranium nephrotoxicity
Q1WIM3	4	Cell adhesion molecule 3		
P01830	4	Thy-1 membrane glycoprotein		
Q63556	3.67	Serine protease inhibitor A3M		
Q62930	3.36	Complement component C9		
O08628	2.55	Procollagen C-endopeptidase enhancer 1		
P32038	2.44	Complement factor D		
P15399	2.3	Probasin		
P26051	1.89	CD44 antigen		
Q62740	1.8	Secreted phosphoprotein 24		
P14046	−1.64	Alpha-1-inhibitor 3		
P10111	−1.75	Peptidyl-prolyl cis-trans isomerase A		
P07314	−1.77	Gamma-glutamyltranspeptidase 1		
Q6DGG1	−1.91	Protein ABHD14B		
P28826	−1.98	Meprin A subunit beta		
Q9QZH0	−2.12	Frizzled-4		
P04764	−2.25	Alpha-enolase		
Q9JHY1	−2.61	Junctional adhesion molecule A		
Q08464	−2.75	Frizzled-2		
Q99J86	−3.00	Attractin		
Q63691	−3.11	Monocyte differentiation antigen CD14		
P97546	−3.21	Neuroplastin		
O35112	−3.33	CD166 antigen		
Q642A7	−3.83	Protein FAM151A		
P04904	−4.20	Glutathione S-transferase alpha-3		
P63102	−4.60	14-3-3 protein zeta/delta		
Q6AXR4	−5.20	Beta-hexosaminidase subunit beta		
Q6AYT0	−5.25	Quinone oxidoreductase		
P80254	−5.49	D-Dopachrome decarboxylase		
O35244	−5.59	Peroxiredoxin-6		

(continued)

Table 18.1 (continued)

UniProt	Fold change	Description	Candidate biomarker	Related diseases
Q9R0D6	−5.66	Transcobalamin-2		
O35217	−5.71	Multiple inositol polyphosphate phosphatase 1		
Q6P6V0	−6.49	Glucose-6-phosphate isomerase		
Q2TL32	−6.99	E3 ubiquitin-protein ligase UBR4		
Q62786	−6.99	Prostaglandin F2 receptor negative regulator		
Q9Z0W7	−7.66	Chloride intracellular channel protein 4		
P62959	−8.00	Histidine triad nucleotide-binding protein 1		
Q9WUK5	−9.00	Inhibin beta C chain		
Q68FQ2	−9.00	Junctional adhesion molecule C		
P19468	−9.33	Glutamate-cysteine ligase catalytic subunit		
O08557	−10.00	N(G),N(G)-dimethylarginine dimethylaminohydrolase 1		
Q66H12	−11.00	Alpha-N-acetylgalactosaminidase		
Q562C9	−17.00	1,2-Dihydroxy-3-keto-5-methylthiopentene dioxygenase		
Q64602	−18.50	Kynurenine/alpha-aminoadipate aminotransferase, mitochondrial		
Furosemide				
P02781	8.2	Prostatic steroid-binding protein C2		
P07647	6.2	Submandibular glandular kallikrein-9	Yes	FSGS, adriamycin nephropathy and Thy1.1 glomerulonephritis
P02782	7.6	Prostatic steroid-binding protein C1		
P02780	9.6	Secretoglobin family 2A member 2	Yes	Adriamycin nephropathy and Thy1.1 glomerulonephritis
P22283	4.7	Cystatin-related protein 2	Yes	Adriamycin nephropathy and Thy1.1 glomerulonephritis
P08721	−7.4	Osteopontin	Yes	Kidney injury
Q01177	−3.0	Plasminogen	Yes	FSGS, acute pediatric appendicitis

(continued)

Table 18.1 (continued)

UniProt	Fold change	Description	Candidate biomarker	Related diseases
Spirolactone				
P06866	5	Haptoglobin	Yes	T2DM, acute kidney injury
P81828	−3.9	Urinary protein 2	Yes	Adriamycin nephropathy and Thy1.1 glomerulonephritis
P81827	−7.3	Urinary protein 1	Yes	Adriamycin nephropathy and Thy1.1 glomerulonephritis
P10960	−4.0	Sulfated glycoprotein 1	Yes	Adriamycin nephropathy and Thy1.1 glomerulonephritis
Q09030	−8.5	Trefoil factor 2	Yes	Feline idiopathic cystitis
Hydrochlorothiazide				
P97580		Beta-microseminoprotein		
O35568		EGF-containing fibulin-like extracellular matrix protein 1		
Pentobarbital sodium				
P00758	−3.3	Kallikrein-1		
Q5XI43	−3.1	Matrix-remodeling-associated protein 8		
P15083	−2.6	Polymeric immunoglobulin receptor		
P32038	3.9	Complement factor D		
P10959	4.6	Carboxylesterase 1C		
P20761	9.1	Ig gamma-2B chain C region		
P50123	−2.5	Glutamyl aminopeptidase		
P26051	−2.9	CD44 antigen		
P98158	−3.6	Low-density lipoprotein receptor-related protein 2		
P28826	−10.9	Meprin A subunit beta		
P29598	−2.7	Urokinase-type plasminogen activator		
P17475	8.5	Alpha-1-antiproteinase	Yes	Null
P07154	−4	Cathepsin L1	Yes	Null
P07522	−3.4	Pro-epidermal growth factor	Yes	Null
P27590	−7	Uromodulin	Yes	Null
P02770	5.5	Serum albumin	Yes	Null
P12346	6.8	Serotransferrin	Yes	Null
Q62867	−3.3	Gamma-glutamyl hydrolase	Yes	Null
P15684	−5.7	Aminopeptidase N	Yes	Null
P36373	−3.5	Glandular kallikrein-7, submandibular/renal	Yes	Null
Q64230	−3.6	Meprin A subunit alpha	Yes	Null
Q64319	−4.5	Neutral and basic amino acid transport protein rBAT	Yes	Null
Chloral hydrate				

(continued)

Table 18.1 (continued)

UniProt	Fold change	Description	Candidate biomarker	Related diseases
P17475	8	Alpha-1-antiproteinase	Yes	Null
P07154	−8.9	Cathepsin L1	Yes	Null
P07522	−5.4	Pro-epidermal growth factor	Yes	Null
P27590	−3.7	Uromodulin	Yes	Null
P02625	−12.5	Parvalbumin alpha	Yes	Null
P07632	4.9	Superoxide dismutase [cu-Zn]	Yes	Null
Q9R0T4	−3.5	Cadherin-1	Yes	Null
Q9JJ40	−3.2	Na(+)/H(+) exchange regulatory cofactor NHE-RF3	Yes	Null
P00758	−7.5	Kallikrein-1		
Q5XI43	−9.3	Matrix-remodeling-associated protein 8		
P15083	−3	Polymeric immunoglobulin receptor		
Q6DGG1	8	Alpha/beta hydrolase domain-containing protein 14B		
Q6IRK9	4.8	Carboxypeptidase Q		
P08649	14	Complement C4		
P61972	6.5	Nuclear transport factor 2		
Q920A6	4.7	Retinoid-inducible serine carboxypeptidase		
P82450	9.4	Sialate O-acetylesterase		
P02650	−5.2	Apolipoprotein E		
P31211	−3.6	Corticosteroid-binding globulin		
P08460	−4	Nidogen-1 (fragment)		
Q63083	−16.7	Nucleobindin-1		
P83121	−3.3	Urinary protein 3		
P05371	−5.8	Clusterin		
Prazosin				
P02761	3.2	Major urinary protein	Yes	Acute renal failure
P14668	−2	Annexin A5	Yes	Acute renal failure
P07151	2	Beta-2-microglobulin	Yes	Acute tubular injury
B0BNN3	3.5	Carbonic anhydrase 1	Yes	Bladder cancer
P10758	2.1	Lithostathine	Yes	Dents disease
P06866	3.4	Haptoglobin	Yes	Diabetic nephropathy
P01015	2.3	Angiotensinogen	Yes	Primary hypertension
Q64319	−2	Neutral and basic amino acid transport protein rBAT	Yes	Protein excretion with sodium loading
Q9R0J8	1.9	Legumain		

(continued)

Table 18.1 (continued)

UniProt	Fold change	Description	Candidate biomarker	Related diseases
P26772	2	10 kDa heat shock protein, mitochondrial		
P19223	2.3	Carboxypeptidase B		
P19999	2.4	Mannose-binding protein A		
P14046	-2	Alpha-1-inhibitor 3		
Q9WTW7	−3.3	Solute carrier family 23 member 1		
Q06496	−2.5	Sodium-dependent phosphate transport protein 2A		
Q64602	−2	Kynurenine/alpha-aminoadipate aminotransferase, mitochondrial		
P70502	−2	Solute carrier organic anion transporter family member 1A3		
O35913	−2	Cluster of solute carrier organic anion transporter family member 1A4		
O08839	−2	Myc box-dependent-interacting protein 1		
Q71MB6	−2.5	Solute carrier organic anion transporter family member 4C1		
A4KWA5	−2	C-type lectin domain family 2member D2		
Arginine vasopressin (10 ng/h)				
P06866	2.33	Haptoglobin	Yes	Null
P00786	1.88	Pro-cathepsin H	Yes	Null
P42854	−1.7	Regenerating islet-derived protein 3-gamma	Yes	Null
O70417	1.88	Prolactin-inducible protein homolog		
Q9JHY1	−1.5	Junctional adhesion molecule A		
P19468	−1.7	Glutamate-cysteine ligase catalytic subunit		
Q99J86	−1.8	Attractin		
O88989	−2.2	Malate dehydrogenase, cytoplasmic		
Arginine vasopressin (50 ng/h)				
P08721	6	Osteopontin	Yes	Null
P07171	5.17	Calbindin	Yes	Null

(continued)

Table 18.1 (continued)

UniProt	Fold change	Description	Candidate biomarker	Related diseases
P04797	2.17	Cluster of glyceraldehyde-3-phosphate dehydrogenase	Yes	Null
O35112	1.75	CD166 antigen	Yes	Null
P01026	1.71	Complement C3	Yes	Null
P26644	1.7	Beta-2-glycoprotein 1	Yes	Null
P42854	−2.2	Regenerating islet-derived protein 3-gamma	Yes	Null
Q4QQW8	3.5	Putative phospholipase B-like 2		
Q9JJ40	1.58	Na(+)/H(+) exchange regulatory cofactor NHE-RF3		
Q9WUC4	1.53	Copper transport protein ATOX1		

Null means data were not provided in the original articles. The fold change was acquired by comparing the drug-treated group to the control group

*proteins could be regulated by different medications. 77 of 211 proteins could be adjusted by two or more drugs. 86 proteins are annotated as candidate biomarkers, and of these 86 proteins, 29 can be adjusted by two or more drugs***.** However, only a tiny fraction of the drugs that are commonly used in clinical practice are studied. The influence of other medicine on urinary should be revealed as soon as possible.

On the other hand, we should realize that the effect of drugs on the urine proteome in disease state is likely to be different from that in health. The influence of medications is challenging to balance between the treated and control groups because only patients usually receive medication treatment. However, when urine proteomics information of the therapeutic drug's effect is provided, researchers in the clinical field can predict in advance the characteristics of these drugs upon urine proteome, which will enable them to acquire more reliable results of the pathological mechanisms and biomarkers for specific diseases.

18.4 Diets

Most food consists of mixtures. The active substances from food may interfere with the acid-base balance, hormone secretion, water levels, and electrolyte metabolism in the blood. Thus, dietary intake can affect the filtration and tubular reabsorption of plasma proteins in the kidney. Measuring nutritional exposure markers in the urine are promising supplements to self-reported food intakes. More importantly, dietary differences can be detected in the urine with more sensitivity than in other biofluids such as plasma and saliva (Walsh et al. 2006). Here we summarize the researches about the effects of different diet on urinary proteome.

18.4.1 Beverages

The beverage is the typical drink intake in our daily life, and it is difficult to control the beverage intake before lab test for disease biomarker detection. So it is necessary to find out the influence of common beverage on urinary proteins.

Cola is one of the most popular beverages in our daily life. Tang et al. (2016) studied the effect of cola intake on urinary proteome on rat's LC-MS/MS. After cola intake, 12 proteins significantly changed in urine, including 4 increased and 8 decreased. Six significantly changed proteins were previously annotated as urinary candidate biomarkers. These proteins are parvalbumin alpha, annexin A1, regenerating islet-derived protein3-gamma, Na(+)/H(+) exchange regulatory cofactor NHE- RF3, uromodulin, and CD166 antigen.

Coffee is another favorite beverage worldwide, which is also rich in bioactive compounds (Liu et al. 2016). Ni et al. (Yanyin et al. 2017) analyzed the acute effect of coffee consumption on urinary proteome in ten young healthy individuals. Eleven proteins were found significantly changed in all ten urine samples after coffee intake, indicating that acute coffee intake exerts impacts on urine proteome. Among all differential proteins, metalloproteinase inhibitor 2 was previously annotated as a urinary candidate biomarker of bladder cancer.

Sugar is usually rich in various drinks, and they may increase blood glucose concentration. Li et al. (2015) analyzed the effect of transient blood glucose increases after oral glucose intake on the human urinary proteome. Only the protein neutrophil defensin 1, which was reported to participate in reducing blood glucose levels, was increased in the urine after glucose intake, and almost all urinary proteins may not be affected. Transient increase of blood glucose levels may not be a significant interfering factor in biomarker studies, at least with current levels of identification and quantification.

The human body needs adequate water to function normally, and *water* drinking is an important source. Using 2D-PAGE analysis, Thongboonkerd et al. evaluated the effect of excessive water drinking (1 L within 20 min) on urinary proteome in three subjects. They found that excessive water drinking caused alterations in the urinary proteome profile with newly presenting spots and also proteins with decreased excretion levels. Regrettably, protein identification was not performed. Thus these altered proteins' information is not available. Further work on protein identification is necessary to carry out.

Polyphenol-rich diets have been associated with a reduced risk of cardiovascular disease. The polyphenol is often rich in fruits and juice. Mullen et al. (2011)examined the effect of a polyphenol-rich (P-R) drink for 2 weeks on urinary biomarkers by CE-MS. Levels of 27 polypeptides were more than fourfold different between the two groups, indicating a substantial difference between the placebo and P-R juice drink groups. These peptides originate from seven proteins, which were previously found to be part of a coronary artery disease (CAD) specific urinary biomarker pattern, including a-1-antitrypsin, collagen a-1(I) chain, fibrinogen alpha chain, collagen a-2(V) chain, xylosyltransferase 1, collagen a-2(I) chain, and Ig

kappa chain C region. Data from this study clearly emphasize the potential of urinary proteome analysis in assessing potential effects of food supplements (especially polyphenols) on pathological processes.

18.4.2 Olive Oil

Olive oil (OO) is the primary source of fat in the Mediterranean diet and is reported to reduce the incidence of cardiovascular diseases (Covas 2007). The EFSA (European Food Safety Authority) claims that daily consumption of 5 mg hydroxytyrosol and derivatives (per 20-g OO dose) could protect LDL particles from oxidative damage (Regulation EU 432/2012 2012). OO is increasingly consumed. Sandra et al. (Silva et al. 2015)evaluated the impact of a 6-week supplementation with OOs (20 mL/day) either low or high in phenolics on highly specific urinary proteomic biomarkers. Among the proteins sequenced, six proteins elevated in urine after high-phenolic olive oil intake, including retinol-binding protein 4, collagen a-1(I) chain, uromodulin, collagen a-2(V) chain, a-1-antitrypsin, and collagen a-2(I) chain. Three proteins elevated in urine after low-phenolic olive oil intake, including collagen a-1(I) chain, collagen a-2(VI) chain, and xylosyltransferase 1.

18.5 Summary of This Section

As reviewed in this section, diet can affect urinary proteome, including coffee, cola, water drinking, olive oil, and polyphenol-rich juice. These factors are common in our daily life and needed to be taken into consideration when biomarker researches are carried out. The proteins that can be affected by these factors and their relationship with certain diseases are listed in Table 18.2. In total, 31 proteins could be influenced by these different factors, and 17 proteins are reported as potential biomarkers.

18.6 Spaceflight

Spaceflight is one of the most extreme environments encountered by humans. The physiological impact of human spaceflight missions exceeding several weeks poses problems such as radiation exposure, immunological depression, and stress. The most important of these factors are radiation, microgravity, hypodynamia, and isolation (Williams et al. 2009). Spaceflight disturbs homeostasis in the human body and affects the majority of physiological systems, and long-term exposure to spaceflight can lead to bone demineralization (Ulbrich et al. 2014), muscle atrophy (Allen et al. 2009), cardiovascular disorders (Hatton et al. 2002), immune dysregulation (Crucian

Table 18.2 Details of the identified urinary proteins altered by different diet

UniProt	Fold change	Description	Candidate biomarker	Disease-related
Coke				
P02625	Up	Parvalbumin alpha	Yes	Compound-induced skeletal muscle toxicity
P07150	Down	Annexin A1	Yes	Glomerular injury, interstitial fibrosis, HER2 breast cancer
P42854	Up	Regenerating islet-derived protein 3-gamma	Yes	Membranous nephropathy
Q9JJ40	Down	Na(+)/H(+) exchange regulatory cofactor NHE- RF3	Yes	Protein excretion with sodium loading, aldosteronism
P27590	Up	Uromodulin	Yes	Sepsis-induced acute renal failure, focal segmental glomerulosclerosis and mesangioproliferative glomerulonephritis, preeclampsia
O35112	Down	CD166 antigen	Yes	Type 1 diabetes
P20759	Up	Ig gamma-1 chain C region		
P07897	Down	Aggrecan core protein		
P42123	Down	L-lactate dehydrogenase B chain		
P01026	Down	Complement C3		
P50399	Down	Rab GDP dissociation inhibitor beta		
B5DFC9	Down	Nidogen-2		
Coffee				
Q5T5J6	1.89	Transcriptional protein SWT1		
B4DR69	−2.0	Neuronal PAS domain-containing protein 1		
P07711	−1.5	Cathepsin L1		
P17858–2	−2.1	Isoform 2 of 6-phosphofructokinase, liver type		
P20908	1.95	Collagen alpha-1(V) chain		
P16989	−1.9	DNA-binding protein A		
Q14161–8	2.01	Isoform 8 of ARF GTPase-activating protein GIT2		
P61981	−2.2	14-3-3 protein gamma		
F5GWY5	1.55	Podocalyxin		
P16035	1.54	Metalloproteinase inhibitor 2	Yes	Bladder cancer
B7ZB63	1.6	ADP-ribosylation factor 3		

(continued)

Table 18.2 (continued)

UniProt	Fold change	Description	Candidate biomarker	Disease-related
Glucose				
Q62716	Down	Neutrophil defensin 1	Null	
High-phenolic olive oil				
P02753	Up	Retinol-binding protein 4	Yes	Coronary artery disease
P02452	Up	Collagen a-1(I) chain	Yes	Coronary artery disease
P07911	Up	Uromodulin	Yes	Coronary artery disease
P05997	Up	Collagen a-2(V) chain	Yes	Coronary artery disease
P01009	Up	a-1-Antitrypsin	Yes	Coronary artery disease
P08123	Up	Collagen a-2(I) chain	Yes	Coronary artery disease
Low-phenolic olive oil				
P02452	Up	Collagen a-1(I) chain	Yes	Coronary artery disease
P12110	Up	Collagen a-2(VI) chain	Yes	Coronary artery disease
Q86Y38	Up	Xylosyltransferase 1	Yes	Coronary artery disease
Polyphenol-rich juice				
P01009	Up	a-1-Antitrypsin	Yes	Coronary artery disease
P02452	Up	Collagen a-1(I) chain	Yes	Coronary artery disease
P02671	Up	Fibrinogen alpha chain	Yes	Coronary artery disease
P05997	Down	Collagen a-2(V) chain	Yes	Coronary artery disease
Q86Y38	Down	Xylosyltransferase 1	Yes	Coronary artery disease
P08123	Down	Collagen a-2(I) chain	Yes	Coronary artery disease
P01834	Down	Ig kappa chain C region	Yes	Coronary artery disease

Null means data were not provided in the original articles. The fold change was acquired by comparing the drug-treated group to the control group

and Sams 2009), and sleep disorders (Gundel et al. 1997). Understanding protein alteration induced by spaceflight could be the key to unraveling the mechanisms. Proteomic studies of the spaceflight effects are mostly carried out on various models, including cells, plants, and animals (Grimm et al. 2014). Only a few works focused on the changes in the composition of human biological samples. Here we summarized the changes in the urinary proteome.

Ground-based studies in healthy volunteers were carried out to study the effects of different factors on urinary proteome, including isolation, dry immersion, and others. The isolation experiment is performed as an international crew was isolated in a hermetically closed, confined environment without microgravity or radiation. In the Mars 500 project carried out from 2008 to 2011, the subjects were confined for 105 and 520 days and received standard diet (Morukov et al. 2010). Salt intake was also changed between 6 and 12 g/day according to the program of the experiment. During the 105-day isolation, a total of 600 urine proteins were identified from six healthy volunteers by Larina et al. in 2011 (Larina et al. 2012). Clustering of proteins revealed that these proteins were most closely associated with the regime of sodium intake and were involved in the protein-protein interaction networks, such as the regulation of water-electrolyte balance, bone and muscle tissue remodeling,

blood clotting cascades, cardiovascular system, and immune system. The urinary protein composition did not return to the baseline within 1 week of recovery. In 2015, Khristenko et al. systematically identified and quantified proteins of the urine samples by improved parallel reaction monitoring (PRM) method (Khristenko et al. 2015). The abundance of more than one half of the 2000 identified proteins changes in the course of the experiment. SOM machine learning was used to describe the time trajectories of urinary protein abundance. Proteins were divided into three categories: an early (week 1–6), an intermediate (week 7–11), and a late one (week 12–15) activation. Early protein activation was related to immune response and inflammatory processes, while intermediate activation was related to developmental and proliferative processes, and late activation was related to stress and responses to chemicals.

When the isolation period was sustained to 520 days, high abundance variation in urinary protein abundance was observed, with an average difference of one order of magnitude (Khristenko et al. 2015). Besides, *only seven proteins were constant during the entire observation* (Larina et al. 2015). These permanent proteins were epidermal growth factor, polymeric immunoglobulin receptor, plasma serine protease inhibitor, AMBP, keratin, type II cytoskeletal 1, collagen α1 (VI) chain, and serum albumin. These proteins may be independent markers of the various conditions and processes in healthy humans and that they can be used as standards in the determination of the concentration of other proteins in the urine.

Dry immersion is broadly used in gravitational physiology to simulate the effects of microgravity on various systems of the body during spaceflights (Navasiolava et al. 2011). The duration of immersion experiments with healthy volunteers varies from several hours to 8 weeks. Pastushkova et al. (2014) analyzed urine proteome in 14 healthy volunteers who underwent a 5-day dry immersion. Nine proteins related to cardiovascular system were identified. It was shown that 5-day dry immersion modifies urine proteomic profile indicating renal, endocrine, circulatory, and metabolic changes. The artificial atmosphere is another crucial factor for spaceflights, which can lead to hypercapnia and hypoxemia for a long-time exposure (Baranov 2011). Uromodulin, urocortin3, orosomucoid 1, and kallikrein serve as sensitive and specific urinary markers of hypercapnia (Gozal et al. 2009).

Urine proteomic studies with crew members with actual space missions are also performed recently. The first urine proteome study focused on changes induced by the long flight missions at the international space station (ISS) was carried out in 2013 (Pastushkova et al. 2012*).* Ten astronauts were examined after 169- to 199-day spaceflights. Of the 238 identified proteins, only three proteins (afamin, aminopeptidase, and aquaporin 2) were classified as highly sensitive spaceflight markers. They were only detected from samples of the cosmonauts after the flight. In another research, urine samples from 21 cosmonauts before and after long-duration spaceflight at the ISS were analyzed (Brzhozovskiy et al. 2017). Three periods of urine samples were collected: 6 months before the flight (control) and on days 1 (+1 day) and 7 (+7 day) of the recovery period after landing. The core proteome consists of 50 proteins that are detected in more than 70% of the samples. Twenty proteins changed significantly on +1 day and + 7 day concerning control. Most of these pro-

teins participate in the regulation of the immune system, and some of these proteins are related to stress and response to a stimulus.

Proteomic studies indicated that protein contents from urine are altered in model experiments as well as space crew members. More information could be found in the literature reviewed by Alexey et al. (Starodubtseva et al. 2017) They summarized the changes induced by spaceflight in the human proteome, including tissues and biofluids. It can be expected that omics profiling should serve as the foundation for aerospace medicine and research.

18.7 Exercise

Exercise-induced proteinuria is a well-known phenomenon. Though mechanisms are not entirely elucidated, it was observed that different exercise intensities alter the urinary proteome in different ways (Kohler et al. 2015). Lower-/moderate-intensity exercise mainly results in glomerular proteinuria, which is characterized by the increased excretion of medium-sized proteins. High-intensity exercise can lead to mixed-type proteinuria with elevated amounts of small- and medium-sized proteins.

Because exercise results in proteinuria, the details of altered proteins induced should be addressed and thus help to uncover the underneath mechanism. We summarized urinary proteomics analysis on exercise-induced proteinuria. In a pilot study, Kohler et al. (2009) compared urinary protein profiles of athletes in different types of sports (endurance sport, team sport, strength sport, ten for each group) using 2D-PAGE. Endurance sport resulted in a significant increase of mainly medium-sized urinary proteins such as albumin, prostaglandin-H2 D-isomerase, immunoglobulin kappa chain and alpha-2-glycoprotein 1, gelsolin isoform b fragment, CD 201 antigen, kininogen 1, and clusterin isoform 1. Strength sport led to an increase of low molecular weight proteins (transthyretin, CD 59antigen, GM 2 ganglioside activator, and apolipoprotein A) and fragments from high molecular weight proteins (albumin, transferrin, hemopexin, orIgG fragments). The study provides the first overview of 2D maps of the urinary proteome after different types of exercise. Then the same group expands their research to explore urinary proteins that may represent typical patterns of a sports group (Kohler et al. 2010). They found that 2D-PAGE profiles of athletes at rest did not differ from those of control samples.

On the contrary, after a marathon run, several proteins (hemopexin, albumin, orosomucoid 1, transferrin and carbonic anhydrase 1) were elevated. These proteins can be used as an indication of physiological changes during exercise. They also observed some samples showed individual changes. Thus further studies should be carried to investigate differences from intraindividual and athletes within different training phases. Urinary biomarkers' continuum of physical activity identified by other methods or in different biofluid were summarized by Sampson et al. (2014) and Lindsay et al. (Lindsay and Costello 2017). Their potential clinical utility in the

monitoring of musculoskeletal health and recovery following injury were also discussed.

In summary, exercise has a significant impact on the amount and composition of urine. Research into exercise-related alterations in the urinary proteome is limited, thus giving necessary to investigate exercise-induced urinary protein changes further, as well as providing more reliable and meaningful biomarkers information.

18.8 Smoking

The number of habitual smokers is estimated to more than 1 billion worldwide (Ng et al. 2014). Chronic cigarette smoking is the most critical risk factor for lung and oral cancers, cardiovascular diseases, chronic obstructive pulmonary disease (COPD), and other tobacco-related oral diseases (Lee et al. 2018). Proteomic profiling of blood (Bortner Jr et al. 2011), tissues (Kelsen et al. 2008), and saliva (Jessie et al. 2010) from smokers has improved our understanding of the molecular impact of smoking (Huan et al. 2018; Gao 2014a, b). It has been reported that smoking increases urinary albumin/protein excretion (Orth 2002), induces a decline of renal function (Regalado et al. 2000), and leads to a marked risk of irreversible proteinuria in smokers, including moderate smokers (Halimi et al. 2000), thus making it necessary to reveal urinary proteome changes due to smoking.

In 2009, Airoldi et al. analyzed the proteome of human urine, searching for protein markers of exposure to cigarette smoking for the first time (Airoldi et al. 2009). 2D-PAGE coupled with MALDI-TOF-MS was used to compare the urinary protein patterns of healthy smokers and nonsmokers. Three inflammatory proteins (S100A8, inter-alpha-trypsin inhibitor heavy chain 4, CD59) and two isoforms of pancreatic alpha-amylase were significantly higher in smokers, with only one protein zinc-alpha-2-glycoprotein downregulated. In another research, in-gel digestion coupled with UPLC-MS/MS method was applied to distinguish different urinary proteins between smoking and non-smoking groups (Haniff and Gam 2016). Four unique proteins from the smokers, namely, pancreatic alpha-amylase, pro-epidermal growth factor, protein 4.1, and prostatic acid phosphatase, were found to be potential urinary biomarkers to indicate the smoking status of a person. Due to limitations of the technique used above, only a few differential proteins were identified. It is necessary to apply advanced mass spectrometer or pre-fraction technology to uncover more and reliable proteins associated with smoking.

18.9 Commentary and Future Perspective

The content of urine can be affected by various conditions, including drugs (glucocorticoid, anticoagulants, procoagulants, diuretics, anesthetics, α1-adrenergic receptor antagonist, and arginine vasopressin), diet (cola, coffee, glucose, excessive

water drinking, polyphenol-rich juice, and olive oil), spaceflight (isolation, dry immersion, and long flight missions), exercise (endurance sport, team sport, strength sport, and marathon run), and smoking. These extrinsic factors all have a more or less effect on urine proteome. Moreover, some of these proteins are already reported as candidate biomarkers (summarized in Tables 18.1 and 18.2). Therefore, we recommend that researchers should pay more attention to the effects of extrinsic factors as well as physiological conditions on the biofluids.

There are several concerns to be emphasized: Firstly, it should be noticed that relatively small sample sizes were analyzed in some studies discussed in this review. The conclusions may need to be validated on a larger sample size. Secondly, different sample preparation strategies (fractionation or enrichment) should be utilized for in-depth characterization of the urinary proteome. Some proteins being potential biomarkers might exist in urine at a low level. Conventional technology is likely to detect a few of them. Thirdly, as the specific urinary proteins appeared to be modulated by different drugs or other conditions, it may be difficult to diagnose accurately using one biomarker; a panel of biomarkers may be more specific and predictive. Nevertheless, there are tremendous opportunities in the upcoming urine biomarker era.

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Part IV
Other Treasures

Chapter 19
Exhaled Breath: Another Biomarker Source That Is Complementary to Urine

Yongtao Liu and Youhe Gao

Abstract Biomarkers are measurable changes associated with disease. Exhaled breath provides many signs of bodily changes and has been proposed to be a good early biomarker source because it lacks homeostatic mechanisms and the information contained in the breath complements to other similar body fluids, such as urine and tears. Earlier biomarker detection can provide earlier diagnosis, which can bring about more choices and more time for treatment. The benefit of studying exhaled breath in animal models is that most interfering factors can be avoided and earlier changes in disease may be found.

Keywords Exhaled breath · Animal model · Biomarkers · VOCs · Device

19.1 Introduction

Biomarkers are the measurable changes associated with disease. Exhaled breath accumulates many biological changes and has been proposed to be a good source of early biomarkers because breath lacks the homeostatic mechanisms that maintain bodily stability and remove biomarkers from the blood. The blood, like cerebrospinal fluid, is regulated by the steady-state mechanism of the body, and the components are not easily changed. To maintain normal operation of the brain, the body will transfer away the metabolic waste and substances affecting the brain's steady state at all costs. The waste is discharged into the blood by direct exchange, and the blood then discharges the waste in the form of urine, sweat, exhaled breath, tears, and bile. While urine, exhaled breath, tears, sweat, and saliva are considered bodily waste, they may all be goldmines in the search for early biomarkers. Based on this theory,

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more biomarker studies should focus on samples discarded from the body, as waste is an essential part of body stability.

There are many advantages of using breath as biomarker source. As a biomarker source, breath is complementary to urine. The water-soluble waste molecules in the blood are more likely to be removed in the kidney, while gaseous wastes are more likely to be removed in the lungs. Disease biomarkers may be discharged as at least one type of waste, or both (Gao 2015a). Biomarker panel from both urine and breath can increase the specificity when complicated conditions need to be differentiated. They can also help us to understand the disease more comprehensively.

Like urine was taken as biomarker source of diseases of urinary system, currently breath has more often been studied as source of diseases of respiratory and upper digestive track. More studies should be done to reveal the potential of both sources as better biomarker sources than blood on diseases of all organs, even though they may reflect their respective neighbouring organs more sensitively and accurately. The advantage of breath over urine is that we are rarely short breath, unlike oliguria or anuria which I believe happens more often. We produce breath constantly no matter good or bad.

Like the most difficult part of biomarker discovery in urine is to find association rather than to find changes, the biomarkers' discovery in breath will have the same problem: too many changes make it hard to identify which ones are associated with the disease. Using the same roadmap of urine biomarker discovery, animal models need to be used first to identify changes associated with disease before validation in large number of human samples. For animal experiments, collecting clean samples is essential. It is not as easy as collecting urine from animals, even though both breath and urine are much easier to collect from human beings.

Most of the gaseous wastes are small molecule compounds (molecular weights less than 200), which can easily pass through the alveolar wall into the exhaled breath and are also known as volatile organic compounds (VOCs). Even after drinking a small amount of alcohol, ethanol can easily be detected from the breath. Therefore, there is reason to believe that exhaled breath may also be able to reflect early changes in disease and have the same potential as urine for earlier and more sensitive detection. Despite the advantage of exhaled breath as a better biomarker source, exhaled breath biomarker research can be impeded by the fact that changes in breath are too complicated to deduce factors associated with any particular pathophysiological condition, especially in human samples (Gao 2015b). Although human breath is easier to collect for study than animal breath is, clinical exhaled breath samples are difficult to analyse because of the influence of various factors, such as diet and drugs, which made the study of exhaled human breath samples very difficult to explain. The application of animal models is an important means to studying early biomarkers of disease. The advantage of the animal model is that the diet, water, and environmental factors are very well controlled. All the differences reflected in breath are directly associated with the disease. The samples can be taken very early since the starting point of the disease is known. Pathological examinations can be used to track disease progression in animal models, which is clinically impossible. The changes in breath found in animal model studies can then be validated in studies with clinical samples.

At present, there are only a few studies based on animal models. One of the problems is that exhaled breath from animals, especially the exhaled breath of rats and mice, is difficult to collect. Albrecht (Albrecht et al. 2015) studied the VOCs in the exhaled breath of rats, which were anaesthetized and ventilated with synthetic air via tracheotomy for 24 h. However, anaesthetics could affect the exhaled breath components, just like how the anaesthetics pentobarbital sodium and chloral hydrate have the same effect on the urine proteome (Zhao et al. 2015).

Many candidate biomarkers from breath have been reported. Nitric oxide (NO) is a biomarker that has been reported to be associated with many diseases in exhaled breath, such as asthma (Kharitonov et al. 1994). In addition to NO, exhaled breath contains many types of VOCs. These compounds are usually composed of alkane, an olefin, a lower alcohol, an aldehyde, a ketone, and other aromatic hydrocarbons (Fenske and Paulson 1999; Weisel 2010). Exhaled breath can reflect many lung diseases, including asthma (Rumchev et al. 2004), chronic obstructive pulmonary disease (COPD) (Van Berkel et al. 2010), bronchiectasis (Phillips et al. 2007), cystic fibrosis (Robroeks et al. 2010), and interstitial lung disease (Nagaria et al. 2005; Onozawa et al. 2005; Chim et al. 2005). In total, 1840 VOCs were identified from breath (872), saliva (359), blood (154), milk (256), skin secretions (532), urine (279), and faeces (381) in apparently healthy human individuals (de Lacy Costello et al. 2014).

Therefore, designing a device that can collect exhalation from small animals is the basis for studying the exhaled breath of animal models. Next, we show a device to collect the exhaled breath of small animals under natural conditions and to minimise the VOCs released from the skin while eliminating the effects from faeces and urine. Subsequently, the device is tested using a rat model of papain-induced emphysema.

19.2 A Device to Collect Exhaled Breath in Small Animal Models

A device was designed to collect the exhaled breath of experimental animals, without anaesthesia or tracheotomy and in relatively comfortable conditions. The basic goals of this device design were to not only deliver clean air to the animals but also effectively collect the animals' breaths. The entire device consists of four parts (Fig. 19.1). The first part is the air source module (Fig. 19.1, 10), which provides clean synthetic air for the animal to reduce the impact of pollutants on the sample. The second part is the air transmission and flow rate and pressure control module (Fig. 19.1, 20); their main purpose is to further reduce the high-pressure gas released from the gas cylinder (Fig. 19.1, 21). The gas flow metre controls the gas flow rate (Fig. 19.1, 23), and the stable synthetic air is delivered to the animal through stainless steel or pure copper tubing (Fig. 19.1, 24). If the pressure in the system is too high, the pressure can be released through the port (Fig. 19.1, 22) to stabilise the pressure in the entire system. The third part is the animal module (Fig. 19.1, 30). The module body (Fig. 19.1, 31) is produced with custom-made glass. It is designed according to the size of the experimental animal (Fig. 19.1, 32). The animal head faces inward, and the tail faces outward. In the head of the box, the upper end is the

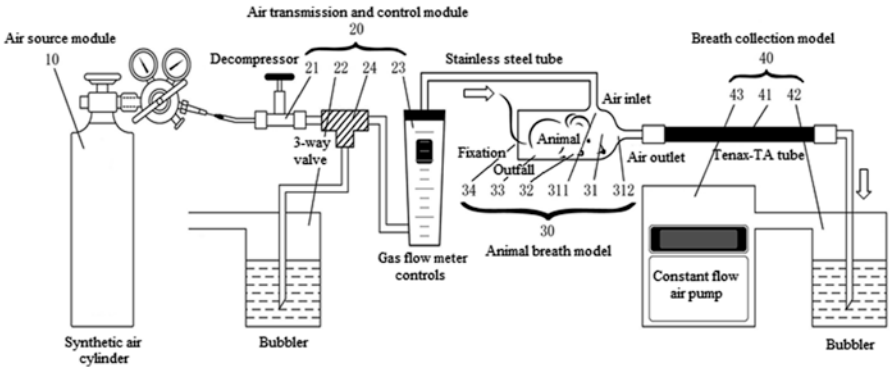

Fig. 19.1 Schematic diagram of the animal breath collection device

inlet for synthetic air (Fig. 19.1, 311), and the front end of the box is the outlet for exhaled breath (Fig. 19.1, 312). A small hole (Fig. 19.1, 33) is opened on the lower side of the hind limb, and a container is placed underneath for collecting animal faeces and urine. At the leftmost part of the module, there is a small hole (Fig. 19.1, 34) where the holder can be vertically inserted to prevent the animal from escaping during the experiment. The fourth part is the exhaled breath collection module (Fig. 19.1, 40), wherein the core component is a Tenax TA tube (Fig. 19.1, 41), and the exhaled breath from the experimental animals is enriched as it flows through. The power source of the gas is provided by the constant flow air pump (Fig. 19.1, 43). The bubbler can provide a clear view of the gas flow (Fig. 19.1, 42). The whole set of equipment does not require electricity (the constant current air pump has a rechargeable battery), which means it is easy to use and has good stability.

The animal storage box is a semi-open environment. When collecting exhaled samples, the animal's own hair can act as a good barrier to prevent contaminated air outside the device from entering the box and contaminating the collection of exhaled samples (Fig. 19.2).

Collecting the exhaled breath of small experimental animals is different from that of large animals for which a mask might be used. Small animals can be placed only in certain spaces to collect the air and enrich the exhaled breath. For small animals, the main difficulty in exhaled breath collection and analysis is that exhaled breath is only a small fraction of the container. (Table 19.1). Table 19.1 shows that the exhaled breath volume of the mice in the unit time was greater than that of the rats. Therefore, when collecting exhaled breath, the exhaled breath collection time of the rats should be appropriately extended.

Notably, part 1 to part 3 of the device can be used separately, and part 4 can be omitted. Replacing part 4 directly connects the device to the online analytical instrument, such as a TD-GC/MS or Q-TOF MS to analyse the VOCs in their exhaled breath. Part 4 is used to store the exhaled breath and can be suitable for use in some situations where online analysis is not appropriate. Although part 4 can store exhaled breath for a short time, it will likely also lose some component information; at the same time, storage conditions need to be considered to prevent contamination of external VOCs (Kang and Paul Thomas 2016; Harshman et al. 2016).

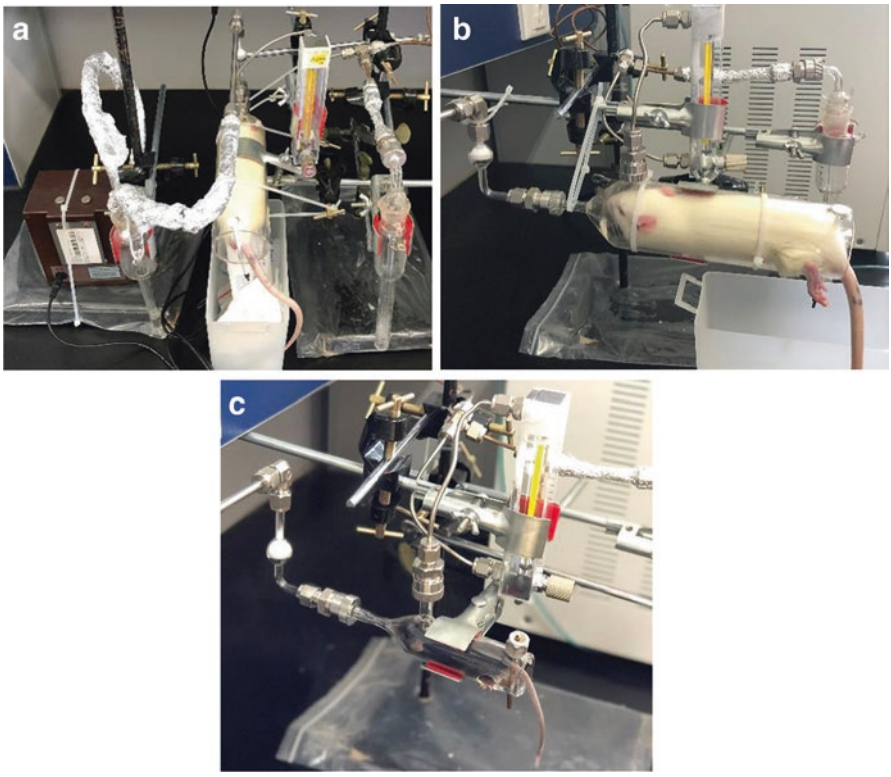

Fig. 19.2 The device is used to collect exhaled breath of experimental animals in a comfortable state. (**a**) SD rat in the device – top view. (**b**) SD rat in the device – side view. **(C)** C57BL/6 mouse in the device – side view

Table 19.1 Respiratory-related physiological parameters in a rodent model and humans

	Mouse	Rat	Human
Respiratory rate (times/min)	163 (84~230)[a]	85.5 (66~114)	15 (12~18)
Heart rate (times/min)	625 (470~780)	475 (370~580)	75 (60~100)
Oxygen consumption ($mm^3/g{\cdot}h$)	1530	2000	250
Ventilation (mL/min)	24 (11~36)	73 (50–101)	7500 (6000~9000)
Tidal volume (mL)	0.15 (0.09~0.23)	0.86 (0.6~1.25)	500 (400~600)

[a]The values stated in the columns refer to mean values, with the typical range in parentheses

We chose a rat model of emphysema to study exhaled breath. SD rats were randomly divided into two groups: the control group and the experimental group. The papain-induced pulmonary emphysema rat model was established as follows [24]. SD rats were anaesthetized by intraperitoneal injection with 2% sodium phenobarbital (40 mg/kg), and papain was instilled through the oral cavity and into the trachea at a rate of 3 mg/100 g of animal weight. In the control group, saline was injected at the same volume. Four rats in the experimental group and two rats in the control group were randomly sacrificed at 2 days, 4 days, 7 days, and 9 days after

injection. Lung tissue samples were quickly fixed in 10% formalin. Then, the samples were embedded in paraffin, sectioned, and evaluated with haematoxylin and eosin (H&E) staining and Masson's trichrome staining.

H&E staining of the lungs obtained from the pulmonary emphysema rats is shown in Fig. 19.3a. On day 3 after papain administration, the alveolar wall is relatively thin, the alveolar wall is composed of a single layer of epithelium, the size of the alveoli is consistent, and a small amount of connective tissue is seen between adjacent alveoli. As time progressed, the alveolar epithelial stroma gradually thickened and partially broke. On day 14, most of the alveolar walls fused and expanded, the microcapsules of different sizes formed, the alveolar epithelium obviously proliferated, the alveolar space obviously thickened, and a small number of inflammatory cells infiltrated. As shown in Fig. 19.3b, Masson's trichrome staining revealed very slight pulmonary emphysema in the lungs on day 3, limited lung interstitial emphysema on day 5, and a large number of collagens fibres on day 14. The dramatic changes in the histopathology of the lung tissue suggest that the pulmonary emphysema model was successful and that typical pulmonary emphysema signs appeared 14 days after papain administration.

Through this animal breath collection device, the exhaled breath from rats in this model of emphysema was successfully collected. In the exhaled breath sample, a total of 220 different VOCs were identified by thermo-desorption-GC-MS (TD-GC-MS, Shimadzu QP2010S). The reproducibility of the overall sample analysis is very good. The retention time of each VOC is very stable. When the split ratio is 10:1, the peak width is narrower and the sample analysis effect is better. Among them, 2-ethyl-1-hexanol was a VOC with a significant change, which decreased in the experimental group on day 5 and was still lower than the control group on day 14 when there was an obvious emphysema lesion (Fig. 19.4 and Fig. 19.5). 2-Ethyl-1-hexanol is an exhaled biomarker that is reported to be closely related to lung cancer (Jia et al. 2018).

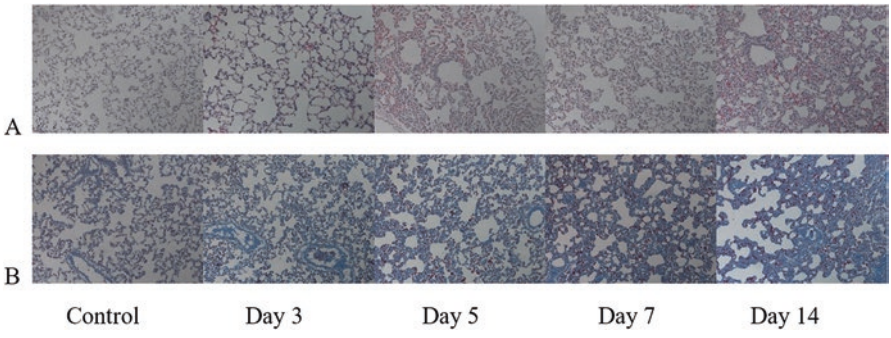

Fig. 19.3 Pathological changes in the lungs of pulmonary emphysema rats. H&E (**a**) and Masson's trichrome staining (**b**) of rat lungs on days 3, 5, 7, and 14 after papain instillation. The magnification was 200x for the images of H&E and Masson's trichrome staining

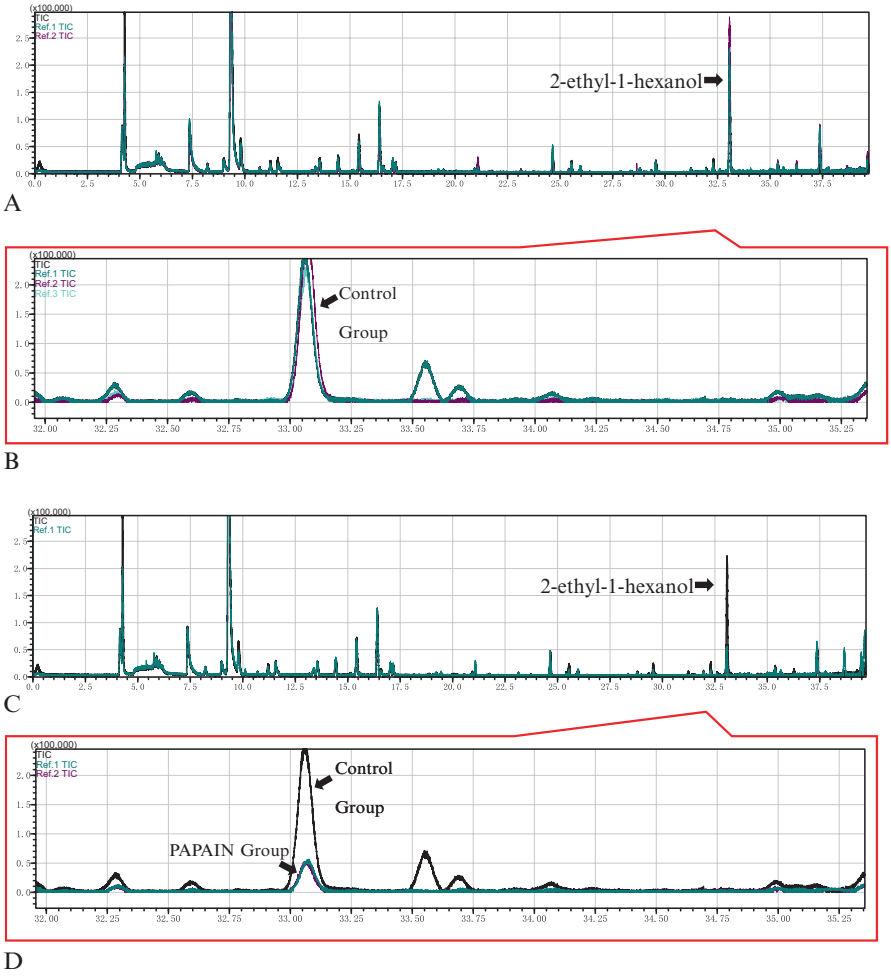

Fig. 19.4 GC-MS spectrum of 2-ethyl-1-hexanol in the exhaled breath of pulmonary emphysema rats. (**a**) TIC of the control group. (**b**) Enlarged view of 2-ethyl-1-hexanol in the control group. (**c**) TIC of the control group and treatment group. (**d**) Enlarged view of 2-ethyl-1-hexanol in the control group and treatment group

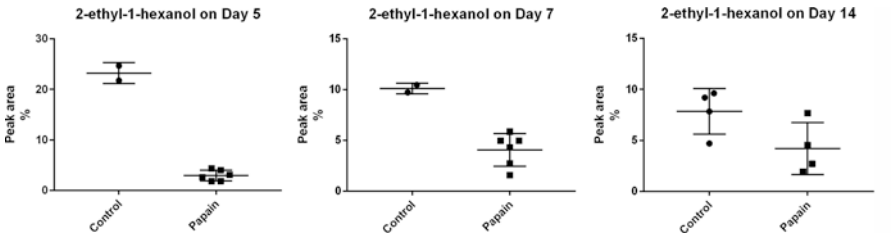

Fig. 19.5 Proportion of the peak area of 2-ethyl-hexanol occupied by exhaled VOCs in a papain-induced pulmonary emphysema rat model at different days (normalised by total peak area)

19.3 Conclusions and Outlook

Unlike urinary samples which can be saved simply and economically on membrane, the storage of breath sample is still challenging. All those fundamental methods need to be developed before breath biomarker discovery studies can be a major choice for most biomarker laboratories. Let's take a deep breath; we will eventually see urine and breath surpass blood as two major biomarker sources in the future. Breath smells good, too.

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Chapter 20
Tears: Potential Window for Monitoring Systemic Conditions

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Abstract Tears covering the ocular surface is an important biofluid containing thousands of molecules, including proteins, lipids, metabolites, nucleic acids, and electrolytes. Tears are valuable resources for biomarker research of ocular and even systemic diseases. For application in biomarker studies, tear samples should ideally be stored using a simple, low-cost, and efficient method along with the patient's medical records. For this purpose, we developed a novel Schirmer's strip-based dry method that allows for storage of tear samples in vacuum bags at room temperature. This dry method facilitates sample transportation and enables the storage of tear samples on a large scale, increasing the availability of samples for studying disease biomarkers in tears. Using this method, tear protein patterns can also be preserved. Liquid chromatography-mass spectrometry/mass spectrometry analysis of proteins recovered from the dry method and traditional wet method showed no significant difference. Some tissue-/organ-enriched proteins were identified in tear; thus tear might be a good window for monitoring the change of these tissues or organs.

Keywords Tear proteome · Biobanking · Biomarkers

20.1 Introduction

Tears overlay the epithelial cells of the cornea and conjunctiva surface. It provides lubrication, protection, and nutrition to the ocular surface. Tear is a complex extracellular fluid, with normal human tears consisting of 1543 proteins (Zhou et al.

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2012), approximately 100 different types of small molecule metabolites (Zhou et al. 2012; Chen et al. 2011), and more than 600 lipid species from 17 major lipid classes (Lam et al. 2014). Tear fluid can be easily and noninvasively accessed (Quah et al. 2014) and has become a useful resource for biomarker research of ocular and systemic diseases. According to a recent review (Hagan et al. 2016), hundreds of potential specific molecular biomarkers in tears were found to be associated with ocular diseases such as dry eye disease, keratoconus, and Graves' orbitopathy. Other reports showed that tears can also reflect the states of breast cancer, prostate cancer, and multiple sclerosis (Hagan et al. 2016; Pieragostino et al. 2015a; von Thun Und Hohenstein-Blaul et al. 2013). For example, Böhm et al. reported a distinctive difference in 20 biomarkers of breast cancer, versus healthy controls (Böhm et al. 2012). Lebrech et al. reported significant differences in tear proteins between breast cancer patients and healthy control, showing 90% specificity and sensitivity (Lebrecht et al. 2009a). It is also reported a panel of 20 biomarkers with an overall specificity and sensitivity of 70% (Lebrecht et al. 2009b). Moreover, tears may reflect central metabolism in some neurological disorders such as multiple sclerosis (Pieragostino et al. 2015a).

Tears show promise as biofluids for biomarker studies and should be preserved along with a patient's medical record. This is a critical step in validation, which facilitates biomarker research and its translation from the bench to the bed. The primary methods for collecting tears are using the Schirmer's strip and glass capillary tube, followed by flash freezing at −80 °C (Zhou and Beuerman 2012). Cryopreservation of tears cannot absolutely prevent the degradation of proteins, as the samples contain various enzymes and hydrolases. Additionally, use of the required cold chain during sample transportation is challenging and costly.

Here, we dried the Schirmer's strip soaked with tears and stored the strip in a vacuum bag. Importantly, the proteins were dry, preventing their degradation and enabling preservation at room temperature.

20.2 Methods of Tear Samples Biobanking

Reported methods used for collection, extraction, and storage of tear samples were compared comprehensively in a recent review (Pieragostino et al. 2015b). In brief, there are two main techniques for tear collection, the Schirmer's strip approach and the glass capillary approach, with the former being widely used. To avoid degradation of the proteins catalyzed by the protease in the tears, the Schirmer's strips were stored at −80 °C, either before or after protein extraction. We recently developed a novel Schirmer's strip-based dry method that allows for storage of tear samples in vacuum bags at room temperature (Weiwei et al. 2017). By using our technique, tear proteins could be stored at room temperature for a fairly long time without consideration of sample loss, which in the long run may greatly facilitate clinical usage. A summary of all these methods was shown as follows (Table 20.1).

Table 20.1 Summary of tears collection and storage methods

Method	Description	Advantages	Disadvantages
Schirmer's strip A	The strip is immediately immersed in an extraction buffer containing protease inhibitor, centrifuged, and snap frozen at −80 °C	Protease inhibitor preserve sample during storage	Time occurring between sampling and extraction could be different
Schirmer's strip B	The strip is snap frozen at −80 °C and afterwards extracted	Good reproducibility in the extraction procedure	Proteins could be degraded because no protease inhibitor are added
Schirmer's strip C (dry method)	The strip is immediately dried and stormed in a vacuum bag at room temperature	Stormed at room temperature without degradation	The procedure is not standardized
Glass capillary	Tears are collected in the tube, centrifuged, and snap frozen	Minimal cell contamination	Not routinely, difficult to realize

We first reported the dry method for preserving tear samples. Compared to the wet method (Schirmer's strip A/B, glass capillary), the most significant difference between these two methods is on preservation procedure. By the wet method (the primary method), after the tear collection, the Schirmer's strip is flash freezing at −80 °C. The disadvantage is that cryopreservation of tear samples cannot absolutely prevent the degradation of proteins, as the samples contain various enzymes and hydrolases. Additionally, use of the required cold chain during sample transportation is challenging and costly. By the dry method, after the tear collection, the Schirmer's strip is dried and stored in a vacuum bag. The advantage is that the proteins were dry, preventing their degradation and enabling preservation at room temperature. Therefore, higher dryness degree and vacuum degree should keep tear samples at room temperature for longer period.

20.3 The Dry Method for Preserving Tears

Tear collection and preservation: Tear samples were collected from volunteers using Schirmer's strips (Tianjin JingMing New Technological Development Co., Ltd) without local anesthesia. The Schirmer's strips were placed at the lateral 1/3 of the lower conjunctival sacs of both eyes for 5 min, and strips with tears exceeding 10 mm were collected. The strips were dried immediately with a hair dryer (Philips HP8200) 70 °C for 1 min and then stored in properly labeled aseptic plastic bags, respectively. The bag was then sealed using a kitchen vacuum sealer and stored at room temperature.

Protein extraction: The strip was cut into small pieces and transferred into a 0.6 mL tube. Next, 200 μL elution buffer (100 mM NH3HCO3, 50 mM NaCl) was added and gently shaken for 2 h at room temperature. The tube was punctured at the

bottom with a cannula, placed in a larger tube (1.5 mL), and centrifuged at 12,000 g for 5 min (Posa et al. 2013). The filtrate in the outer tube was collected and quantified by the Bradford method.

In addition to proteins, other biomolecules in tears were preserved on the strip by the dry method, including lipids, metabolites, nucleic acids, and electrolytes. Since all tears were soaked on the strip, only water and some volatile matter was lost during the drying procedure.

20.4 Tears May Serve as a Window for Systemic Conditions

Recently, Uhlén et al. reported a tissue-based map of the human proteome, describing the expression and distribution of human proteins across 44 different tissues and organs, both at the mRNA (32 tissues) and protein level (Uhlen et al. 2015). In our study, we identified 514 tear proteins, and then we compared each of them to the tissue-enriched proteome. In all, 365 proteins that are highly enriched in different tissues and organs were also identified in tears, and 132 proteins corresponding to 132 protein-encoding genes that highly are expressed in different tissues and organs were also detected in tears (Fig. 20.1). This is an observation that tissue-/organ-enriched proteins are present in tear. There is no known mechanism as far as we know. At this point, we can only propose the possibility that if those organs had functional and/or structural changes, proteins enriched in those organs may be released in a different quality and quantity into the blood. These changes may somehow reach in tear and be reflected on proteins in tear. Therefore, tear might be a good window for monitoring the change of these tissues or organs. These proteins are not specific to those organs. They may also be made by tear gland locally.

On the other hand, intraocular inflammation appeared not to cause significant proteomic changes in tears. In our recent study, tear proteomes between the diseased and the contralateral quiescent eye in Behcet's disease-associated uveitis (BDU) with unilateral relapse were analyzed using data-independent acquisition (DIA) strategy (Liang et al. 2018). Unexpectedly, severe relapse of unilateral intraocular inflammation did not appear to cause asymmetric tear proteome changes. Thus, tear proteome may not reflect pathologic changes in intraocular diseases, but our results did not preclude tears as a valuable source for biomarker studies of systemic diseases.

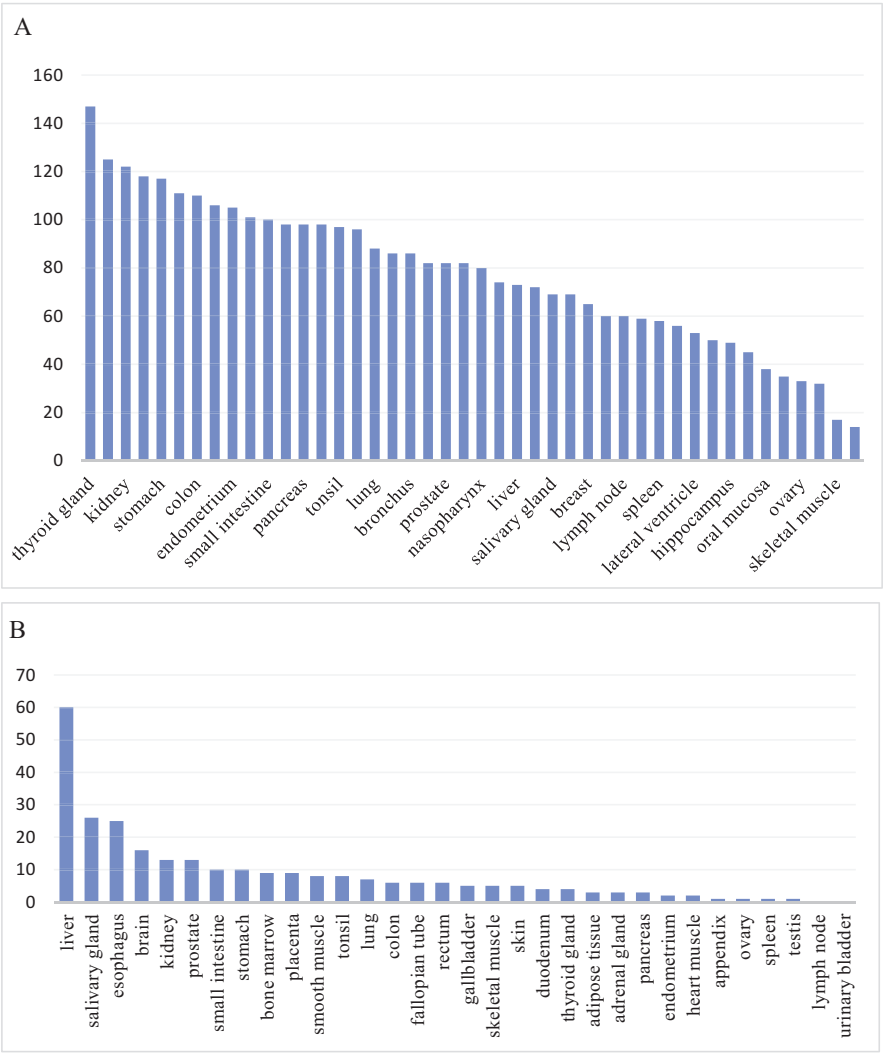

Fig. 20.1 Tissue distribution of tear proteins. Tear proteome distributions across 44 tissues on protein level (**a**) and mRNA level (**b**)

20.5 Conclusions and Future Prospects

Tears might be another promising biofluid for discovery of disease-associated biomarkers in addition to urine. However, there is still a long way to go before tears can be widely used in clinical application. Continuous efforts are needed to improve methods of tear preservation and analysis, and a huge number of clinical studies are needed to excavate valuable biomarkers for a variety of systemic diseases considering the substantial heterogeneity of tear components.

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