The potential urinary aging markers for 20-month-old rats

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ABSTRACT:

Urine is a very good source for biomarker discovery because it accumulates the changes of body. The urinary proteome is influenced by various factors, which is a major challenge in urinary biomarker discovery. To circumvent these problems, simpler systems, such as animal models, should be used to establish associations between physiological or pathological conditions and changes in the urinary proteome. In this study, the urinary proteome of young (2-month-old) and old rats (20-month-old; 9 in each group) were analyzed using LC-MS/MS and quantified using the Progenesis LC-MS software. A total of 371 proteins were identified, 194 of which were shared between young and old rats. Based on the criteria of a fold change ≥ 2, $P < 0.05$ and being identified in each rat in the high abundance group, 33 proteins were changed (15 up-regulated and 18 down-regulated in old rats). By adding a more stringent standard (protein spectral counts from every rat in the higher group greater than those in the lower group), 8 proteins were changed consistently in all rats of between the groups, 2 of which are also altered in the urinary proteome of aging humans. There are no shared proteins between our results and the previous aging plasma proteome. Twenty of the 33 (60 %) changed proteins have been reported to be disease biomarkers, which implies that aging may share similar urinary changes with some diseases. The 33 proteins corresponded to 28 human orthologs, which are strongly expressed in the kidney, intestine, cerebellum and lung, according to the human protein ATLAS. Therefore, the urinary proteome may reflect aging conditions in these organs.

INTRODUCTION

Biomarker is the measurable change associated with a physiological or pathophysiological process. Urine can accumulate lots of changes in the body and is thus likely to be a good biological source for disease biomarker discovery(Gao 2013; Gao 2014). As summarized in a recent paper(Gao 2015a), many physiological changes can be reflected in urine, so we have reason to expect that urine can be used to detect small, early changes in pathological and/or pharmacological conditions(Gao 2015b). Urine can be more sensitive to detected changes than blood (Li 2014). In some previous biomarker studies(Huang et al. 2012; Wu et al. 2013), several potential biomarkers perform even better in urine than in blood(Youhe 2014). Therefore, urine is an ideal source for biomarker discovery, and many urinary biomarkers have been reported in various types of diseases (Shao et al. 2011).

The major challenge in urinary biomarker discovery is that the urinary proteome can be affected by a variety of factors, such as gender(Guo et al. 2015), age, medications(Li et al. 2014; Zhao et al. 2015), exercise(Kohler et al. 2009), smoking(Airoldi et al. 2009), and many other physiological variables and environmental factors. To identify specific urinary associations with a particular disease in clinical samples, we must eliminate or balance these factors as much as possible by using a large sample size in human urinary biomarker studies. Changes in stable components of the urine proteome are more likely to be disease biomarkers(Sun et al. 2009). Therefore, a better understanding of the effect of physiological and environmental factors on the urine proteome will facilitate urinary biomarker discovery. To achieve this objective, it is essential to introduce much simpler and more controllable systems, such as animal models. Animal models have similar genetic backgrounds and the same living environment; thus, it is easy to use a small sample size to establish associations between physiological or pathological conditions and the corresponding changes in urine. Therefore, screening potential disease biomarkers in animal models followed by validation in human samples may be a good strategy for urinary biomarker discovery.

Aging is a complex physiological process that induces a decline of function in multiple organs. Urine is a window into the body, so the urinary proteome should reflect the pathophysiological changes and aging conditions in many organs. For example, urinary age-related peptide excretion patterns may allow the non-invasive detection of renal disease and show high resemblance between human aging and human chronic kidney diseases(Zurbig et al. 2009). Urinary proteome changes in the elderly appear to reflect the physiological processes of aging and are particularly clearly represented in the circulatory and immune systems(Bakun et al. 2014). Therefore, a detailed analysis of the urinary proteome may be informative of the physiological changes associated with aging.

Urine proteomics has become a non-invasive, reproducible method that is easy to repeat at high frequencies. In the current study, the urinary proteomes of young (2-month-old) and old (20-month-old, n=9) rats were analyzed using LC-MS/MS. We identified age-specific urinary proteins and the urinary proteome may reflect aging conditions in many organs.

MATERIALS AND METHODS

Animals and ethics statement

Pathogen-free, male Sprague-Dawley rats were purchased from the Institute of Laboratory Animal Science, Chinese Academy of Medical Science & Peking Union Medical College (Beijing, China). All animals were fed a standard laboratory diet and kept under controlled temperature (22 ± 1 ℃) and humidity (65–70%). The study was performed after the rats had been allowed to acclimate for 1 week. This study was approved by the Institute of Basic Medical Sciences Animal Ethics Committee, Peking Union Medical College (Animal Welfare Assurance Number: ACUC-A02-2013-015). All rats received humane care in compliance with the institutional animal care guidelines approved by the Institutional Animal Care and Use Committee of the Peking Union Medical College.

Sample collection and preparation

Nine young rats (2 months of age) and 9 old rats (20 months of age) were allowed to acclimate for 1 week. The rats were all weighed, and urine samples were collected using metabolic cages. Urinary protein and creatinine concentrations were measured at the Peking

Union Medical College Hospital. The urine samples were centrifuged at 5000 g for 30 min, and the supernatants were precipitated with 75% v/v acetone for 12 h followed by centrifugation at 12000 g for 30 min. After removing the supernatant, the pellets were thoroughly air-dried, resuspended in lysis buffer (8 M urea, 2 M thiourea, 50 mM Tris and 25 mM DTT) and subjected to protein quantitation using the Bradford assay. Proteins were digested using trypsin (Trypsin Gold, Mass Spec Grade, Promega, Fitchburg, WI, USA) using filter-aided sample preparation methods(Wisniewski et al. 2009). Briefly, urinary proteins were loaded on the filter unit (Pall, Port Washington, NY, USA), denatured at 50 ℃ for 1 h by the addition of 20 mM DTT and alkylated in the dark for 40 min by the addition of 50 mM IAA. Proteins were digested using trypsin (1:50) at 37 ℃ overnight. The digested peptides were desalted using Oasis HLB cartridges (Waters, Milford, MA, USA).

LC-MS/MS

The resulting peptides were analyzed by nanoLC-MS/MS with an Agilent 1200 HPLC system coupled to an LTQ-Orbitrap Velos mass spectrometer (Thermo Fisher Scientific, Bremen, Germany). Each sample was loaded with a maximal volume of 8 μL onto a Michrom Peptide Captrap column (MW 0.5–50 kD, 0.5 × 2 mm; Michrom Bioresources, Billeria, MA, USA) with a flow rate of 20 μL/min in 0.1% formic acid 99.9% water. The trap column effluent was transferred to a reversed-phase microcapillary column (0.1 × 150 mm, packed with Magic C18, 3 μm, 200 Å; Michrom Bioresources) in an Agilent 1200 HPLC system. The elution gradient for the reverse column changed from 95% mobile phase A (0.1% formic acid, 99.9% water) to 40% mobile phase B (0.1% formic acid, 99.9% acetonitrile) over 60 min at a flow rate of 500 nL/min. The LTQ-Orbitrap Velos was operated in data-dependent acquisition mode. Survey scan MS were acquired in the Orbitrap on the 300-2000 *m/z* range, with the resolution set to a value of 60,000. The 20 most intense ions per survey scan were selected for CID fragmentation, and the resulting fragments were analyzed in the LTQ. Dynamic exclusion was employed with a 60-second window to prevent the repetitive selection of the same peptide.

Database searching and protein quantification

The Mascot Daemon software (version 2.4.0, Matrix Science, London, UK) was used to search the MS/MS data against the SwissProt_rat database (release 2013_07; taxonomy: Rattus; containing 9354 sequences). The carbamidomethylation of cysteines was set as a fixed modification. The oxidation of methionine and protein N-terminal acetylation were set as variable modifications. The specificity of trypsin digestion was set for cleavage after K or R, and two missed trypsin cleavage sites were allowed. The mass tolerances in MS and MS/MS were set to 10 ppm and 0.5 Da, respectively. Peptide and protein identifications were validated using Scaffold (version 4.0.1, Proteome Software Inc., Portland, OR, USA). Peptide identifications were accepted if they were detected with ≥95.0% probability by the Scaffold local false discovery rate algorithm, and protein identifications were accepted if they were detected with ≥99.0% probability and contained at least 2 identified peptides(Nesvizhskii et al. 2003). Label-free quantification was performed using the Progenesis LC-MS software (version 4.1, Nonlinear, Newcastle upon Tyne, UK) as previously described(Stoop et al. 2013).

RESULTS AND DISCUSSION

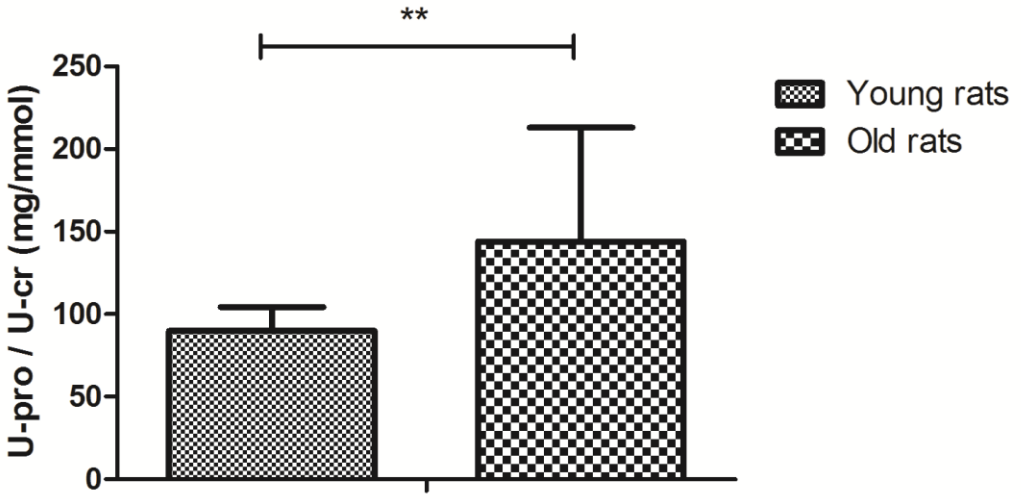

Figure 1 Urine protein-to-creatinine ratio in young and old rats (P <0.001, n = 9 per group)

Urinary protein-to-creatinine ratio is increased in old rats

The urinary protein-to-creatinine (U-pro/U-cr) is an important index for monitoring kidney function. In this study, the U-pro/U-cr value increased approximately 2.2-fold (90±15 in young rats versus 199±60 in old rats, P <0.001; Figure 1), which suggests that kidney function is decreased in old rats.

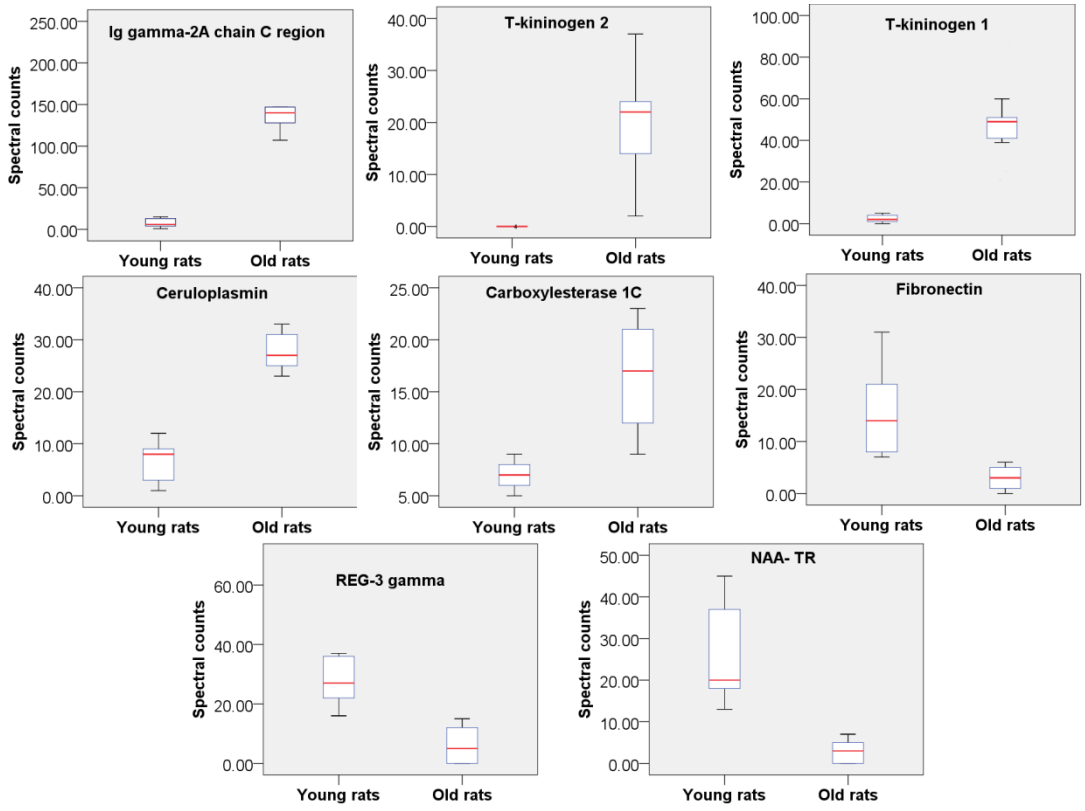

Figure 2 Relative quantitation of 8 urine proteins identified as being related to aging (n=9 per group; P < 0.05 for every protein).

Urinary proteome changes between young and old rats

To investigate how the urine proteome changes with age, 18 LC-MS/MS runs of urine samples in young and old rats (n=9 per group) were performed on an LTQ-Orbitrap Velos. The false discovery rate (FDR) was adjusted to be less than 1%. A total of 371 proteins were identified, 194 of which were shared between young and old rats. All proteins are listed in Table S1.

Based on label-free quantification using the Progenesis LC-MS software, 33 proteins were changed (fold change ≥ 2, $P < 0.05$ and identified in each rat of the high abundance group; Table S2), of which 15 proteins were up-regulated and 18 were down-regulated in old rats, which suggests that the urinary proteome is greatly changed with age. Twenty of the 33 (60%) changed proteins have been reported to be disease biomarkers (Shao et al. 2011) and show high resemblance between aging and certain diseases. It is thus necessary to match the age of the disease and control groups in urinary biomarker research.

To find the most reliable age-related proteins, screening was performed with a stricter standards: protein spectral counts in all rats in the higher abundance group must be greater than those in the lower group. Under these conditions, 8 proteins were significantly changed in old rats and were thus considered age-specific proteins (Figure 2). Five of these proteins were increased in old rats, and 3 were decreased. Two proteins, Ig gamma-2A chain C region and ceruloplasmin, also showed consistent trends in the urine of aging humans in a previous study(Bakun et al. 2014). Urinary proteins that have consistent expression patterns between animals and humans are more reliable age-related proteins. Therefore, screening potential disease biomarkers in animal models and then validating them in human samples may be a good strategy for urinary biomarker discovery.

Human orthologs of the rat proteins significantly affected by aging

It is typically assumed that orthologs (co-orthologs) retain similar functions between species (Koonin 2005). We thus transformed the significantly changed proteins with aging in rats to human orthologs. Based on the 122.R_norvegicus.orthologues database and Ensembl Compare database (Shaye & Greenwald 2011), 25 of the 33 rat urinary proteins corresponded to 28 human orthologs (Table S2). By comparing the proteins with the human core urinary proteome, we further found that 16 human orthologs are relatively stable proteins in the normal human urinary proteome(Nagaraj & Mann 2011; Sun et al. 2009). Therefore, such proteins could serve as potential urinary biomarkers because significant qualitative or quantitative changes to these stable proteins may suggest pathophysiological conditions (Sun et al. 2009).

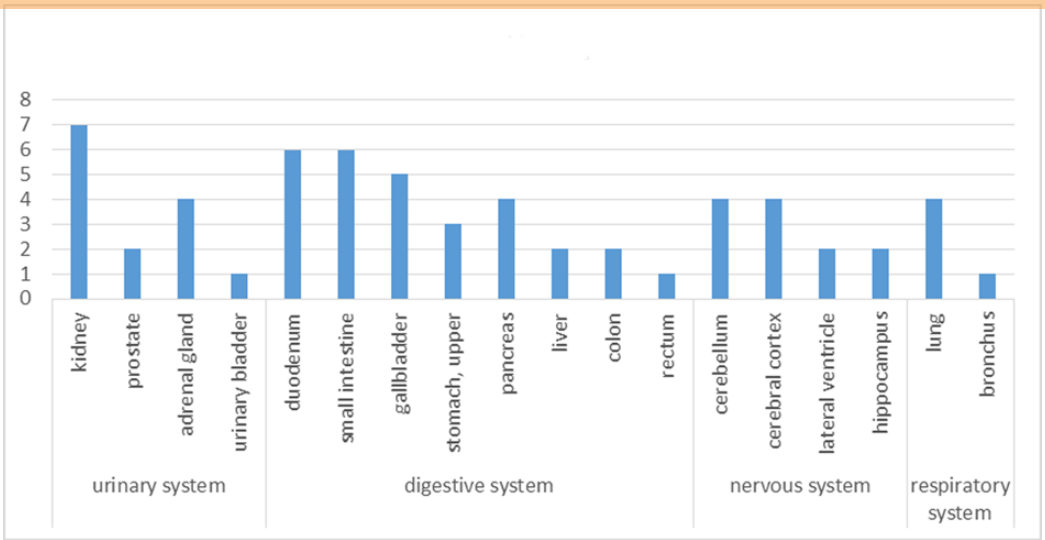

Figure 3 Tissue distribution of the human orthologs of aging-associated rat proteins. X-axis: human tissues; Y-axis: the number of strongly expressed proteins in human tissues compared the human orthologs with The Human Protein Atlas.

The Human Protein Atlas contains histological images obtained using sections from human tissues. Each tissue was examined for representativeness, and immunoreactivity in the different cell types present in normal or cancer tissues was subsequently annotated. Basic annotation parameters included an evaluation of staining intensity (negative, weak, moderate or strong). According to the Human Protein Atlas, significantly changed proteins are strongly expressed in the kidney, intestine, cerebellum and lung (Figure 3). Therefore, the urinary proteome may preferentially reflect the aging conditions of these organs. By improving the depth of the urinary protein identification in future, the conditions of more organs may be reflected in the urinary proteome (Figure S1).

ADDITIONAL INFORMATION AND DECLARATIONS

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Competing Interests

The authors declare no competing interests.

Author Contributions

•Xundou Li performed the experiments, analyzed the data, contributed reagents/materials/analysis tools, wrote the paper, prepared figures and tables.

• Youhe Gao conceived and designed the experiments, reviewed drafts of the paper.

Supplemental Information

Table S1 All the proteins identified and quantified in this experiment.

Table S2 The 79 proteins changed between young and old rats with $P < 0.05$.

Figure S1 All the tissues that aging-associated rat proteins distribute

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