Identification of phosphorylated MYL12B as a potential plasma biomarker for septic acute kidney injury using a quantitative proteomic approach

Fan Wu1,2, Xiu-Juan Dong3, Yan-Yan Li1, Yan Zhao1, Qiu-Lin Xu4,5, Lei Su4,5

1Southern Medical University, Guangzhou 510515, Guangdong, China; Departments of 2Nephrology, 3Hematology, The Third Hospital of Zhengzhou 450000, Zhengzhou, Henan, China; 4Department of Intensive Care Unit, General Hospital of Guangzhou Military Command, Guangzhou 510010, Guangdong, China; 5Key Laboratory of Tropical Zone Trauma Care and Tissue Repair of PLA, Guangzhou 510010, Guangdong, China

Received August 31, 2015; Accepted October 19, 2015; Epub November 1, 2015; Published November 15, 2015

Abstract: Acute kidney injury (AKI) is a common and increasingly encountered complication in hospitalized patients with critical illness in intensive care units (ICU). According to the etiology, Sepsis-induced AKI (SAKI) is a leading contributor to AKI and significantly has very poor prognosis, which might be related to the late detection when the elevation of BUN and serum creatinine (SCr) is used. Many genes are up-regulated in the damaged kidney with the corresponding protein products appearing in plasma and urine. Some of these are candidate biomarkers for more timely diagnosis of SAKI. Therefore, extensive research efforts over this past decade have been directed at the discovery and validation of novel SAKI biomarkers to detect injury prior to changes in kidney function, a number of serum and urinary proteins, including NGAL, KIM-1, cystatin-C, IL-18, and L-FABP, have been identified for predicting SAKI before a rise in BUN and serum creatinine in several experimental and clinical trainings. Unfortunately, an ideal biomarker of SAKI with highly sensitivity and specificity has not been identified yet. Recent progresses in quantitative proteomics have offered opportunities to discover biomarkers for SAKI. In the present study, kidney tissue samples from SAKI mice were analyzed by two-dimensional differential gel electrophoresis (2D-DIGE), and 4 up-regulated proteins, which were actin (ACTB), myosin regulatory light chain 12B (MYL12B), myosin regulatory light polypeptide 9 (MYL9), and myosin regulatory light chain 12A (MYL12A) were identified by matrix assisted laser desorption ionization-time of flight/time of flight mass spectrometry (MALDI-TOF/TOF MS). Among all the varied proteins, MYL12B was validated by western blot. Interestingly, there was no change between the SAKI and control kidney tissues, however, phosphorylated MYL12B was detected to be consistent with the proteomics data. Furthermore, phosphorylated MYL12B was found similarly to be increased in SAKI plasma, while MYL12B was changeless in plasma of control group. Taking together, phosphorylated MYL12B may be employed as a potential plasma biomarker for the early diagnosis of SAKI.

Keywords: SAKI, plasma, biomarker, proteomic, phosphorylation, MYL12B

Introduction

Acute kidney injury (AKI) is a common and severe problem in the intensive care units (ICU) in the last decade [1]. The estimated incidence of AKI is 2 to 3 cases per 1,000 persons [2], and has increased in recent years, both in the community and in hospital settings [3, 4]. Between 5% and 20% of critically ill patients in ICU have an episode of AKI, with acute tubular necrosis (ATN) accounting for about 75% of cases [5, 6]. Despite significant advances in both critical care and nephrology, the mortality rate of hospitalized patients with AKI has remained relatively unchanged at around 50% over the past few decades [7]. The most important contributing factors for AKI in hospitalized patients is sepsis, the combination of sepsis and AKI portends a dire clinical situation that is associated with a hospital mortality rate as high as 70% [8, 9]. Sepsis-induced AKI (SAKI) is a high burden of morbidity and mortality in both children and adults with critical illness [10]. Various pathophysiological studies have been proposed to describe SAKI, including vasodilation that induces glomerular hypoperfusion, inflammatory process, oxidative...
Phosphorylated MYL12B as a potential plasma biomarker for SAKI

injury and tubular dysfunction [11]. The exact mechanism involved in sepsis-induced AKI is still not clearly understood, due to difficulties to obtain histological data or biochemical kidney function marks in the different sepsis phases in clinical studies [12]. A considerable number of novel biomarkers for SAKI were developed during recent years and tested in clinical trials, such as, cystatin c, neutrophil gelatinase-associated lipocalin (NGAL), interleukin-18 (IL-18), kidney injury molecule-1 (KIM-1), and liver-type fatty acid binding protein (L-FABP), et al. however, very little of value for earlier prognostication has transpired to date [13]. Therefore, screening of ideal biomarkers with highly sensitivity and specificity for SAKI becomes very important and urgent.

Experimental studies involving animal models are fundamental to better define and characterize the pathophysiological phases of SAKI. The cecal ligation puncture (CLP) technique, which is widely used for sepsis induction in animal models, was used in our experiment. CLP is characterized by the distal ligation of the ileocecal valve and needle puncture and ligated cecum cause leakage of fecal contents into the peritoneum of the animal, subsequent to bacteremia and sepsis [12].

Recent progresses in quantitative proteomics have offered opportunities to discover biomarkers for SAKI. Differential in-gel electrophoresis (DIGE), which can co-detect numerous samples in the same two-dimensional gel (2DE) to minimize gel-to-gel variation and compare the protein features across different gels through an internal standard [14], has been widely used to search biomarkers for many diseases.

In this study, we utilized a proteomics-based approach that involving 2D-DIGE analyses, and subsequent MALDI-TOF/TOF MS identification to obtain a panel of differentially expressed proteins from kidney tissues of SAKI mice model. Additionally, one of the varied proteins was validated by Western blot, both with kidney tissues and plasma samples.

Materials and methods

Chemicals and reagents

Generic chemicals were purchased from Sigma-Aldrich (USA). Reagents for 2D-DIGE were purchased from GE Healthcare (USA). Anti-MY-L12B antibody (ab137063), anti-Mylc2b (phospho S18) antibody (ab63479), and anti-rabbit secondary antibody (ab191866) were purchased from Abcam (UK). All the chemicals and biochemicals used in this study were of analytical grade.

Animal models replication for SAKI

All procedures in this study are in compliance with Ethical Principles of Animal Care and received approval from the Ethics Committee on Animal Experimentation at the Institute of Biological Sciences in Southern Medical University for the use of animals in tests.

The CLP model for polymicrobial sepsis developed by Chaudry et al. [15] was used, with some modifications. In brief, 7-8 week old BALB/c mice, weighing between 18 and 22 grams, divided in the following 2 groups: control group and SAKI group. These were housed under specific pathogen-free conditions with free access to standard rodent food and water. We used six BALB/c mice per group for each experiment. Under general anesthesia, midline laparotomy was performed and the cecum was exposed and ligated distal to the ileocecal valve to prevent bowel obstruction, and the distal part of the cecum was punctured with a 22-gauge needle. A small amount of cecal content was manually extruded from the punctured cecum into the abdominal cavity. In all studies, after returning the cecum into the abdomen, 1 mL of phosphate-buffered saline (PBS) for fluid resuscitation was administered to create a more clinically relevant sepsis model as the standard care for human operations. The abdomen was closed using a single-layer technique. The control group mice were treated identically as the operated mice with the exception of the ligation and puncture of the gut. Under these conditions, all CLP mice showed signs of severe illness within 24 hours after the operation and high lethality after 48 hours.

Samples preparation

kidney were harvested 24 h after treatment, sliced, snap frozen in liquid nitrogen and stored at -80°C until homogenization. The whole blood collection was carried out by puncture of the abdominal aorta and subsequent evaluation of renal function. Plasma was stored at -80°C until use.
The frozen kidney tissue samples were weighed (100 mg/ml lysis buffer) and immediately thawed in phosphate-buffered saline solution on ice. The tissues were washed five times with phosphate-buffered saline solution to remove adhered hemoglobin. The dialyzed samples were resuspended in Lysis Buffer (30 mM Tris-Cl, 7 M urea, 2 M thiourea, and 4% CHAPS, pH 8.5) and incubated on ice for 30 min. The suspensions were sonicated on ice to prevent sample heating, and a total of five 10-second bursts with 30-second pauses were used. The lysates were subsequently centrifuged at 12,000 g for 30 minutes. The proteins were precipitated from the suspension using the 2D Clean-up Kit (GE Healthcare) according to the manufacturer’s instructions and resuspended in Lysis Buffer. And then, the protein concentration was determined using the 2D Quant Kit (GE Healthcare) according to the manufacturer’s instructions. The proteins were aliquoted followed by freezing or freeze-drying. All reagents were obtained from the Sigma Chemical Company unless otherwise noted.

2D-DIGE and gel image analysis

Before performing 2D-DIGE, kidney samples from 6 control and 6 SAKI mice were pooled individually. Approximately 50 μg of protein samples were labeled with 400 pmol of either Cy3 or Cy5 for triplicate comparison on three 2D gels, respectively. To facilitate image matching and cross-gel statistical comparison, 50 μg of pooled proteins (a pool of all samples) was also prepared and labeled with 400 pmol Cy2 as an internal standard for every gel. Thus, the triplicate samples and the internal standard can be run and quantified on multiple 2DEs. At the same time, a preparative gel using 600 μg of pooled protein sample without labeling was prepared. The labeling reactions were performed in the dark on ice for 30 min, and then quenched with a 20-fold molar ratio excess of free L-lysine to dye for 10 min. The differentially Cy3- and Cy5-labeled samples were mixed with the Cy2-labeled samples for rehydration. The rehydration process was performed with immobilized non-linear pH gradient (IPG) strips (pH 3-10 NL, 24 cm), which were later rehydrated by Clyde-labeled samples in the dark at room temperature overnight (at least 12 h). Isoelectric focusing was then performed using an IPGphor III apparatus (GE Healthcare) for a total of 60 kVh at 20°C. Strips were equilibrated in 6 M urea, 30% (v/v) glycerol, 1% SDS (w/v), 100 mM Tris-Cl (pH 8.8), and 65 mM dithiothreitol for 15 min, and then in the same buffer containing 240 mM iodoacetamide for another 15 min. The equilibrated IPG strips were transferred onto 26×20 cm 12.5% polyacrylamide-gels casted between low fluorescent glass plates. The strips were overlaid with 0.5% (w/v) low melting point agarose in a running buffer containing bromophenol blue. The gels were run in an Ettan DALT Six gel tank (GE Healthcare) at 3 Watt per gel at 10°C until the dye front had completely run off the bottom of the gels.

Afterward, the fluorescence 2DE gels were scanned directly between the low fluorescent glass plates using an Ettan™ DIGE Imager (GE Healthcare). This imager is a charge-coupled device-based instrument that enables scanning at different wavelengths for Cy2-, Cy3-, and Cy5-labeled samples. Gel analysis was performed using DeCyder 2D Differential Analysis Software v7.0 (GE Healthcare) to co-detect, normalize, and quantify the protein features in the images. Features detected from non-protein sources (e.g., dust particles and dirty background) were filtered out. Spots displaying 1.5 fold increase or decrease in abundance with a P<0.05 were selected for protein identification.

Protein staining and spot picking

The preparative gel was stained with colloidal Coomassie blue G-250. Bonded gels were fixed in 40% (v/v) ethanol, 10% (v/v) acetic acid for 1.5 h, washed three times (10 min each) with ddH2O, and then incubated in 20% (v/v) methanol, 10% (w/v) ammonium sulfate, 10% (v/v) phosphoric acid, and 0.12% (w/v) Coomassie blue G-250 for 5 to 7 days. No destaining step was required. The stained gels were then imaged on an ImageScanner III densitometer (GE Healthcare), which processed the gel images as TIF format files. Matched spots of interest were picked automatically from the preparative gel by Ettan™ Spot Picker (GE Healthcare).

In-gel digestion and protein identification

The picked spots were destained with 50% acetonitrile (ACN)/100 mM NH4HCO3 for 10 min, dehydrated with 100% ACN for 10 min, and
then dried using a centrifugal concentrator (TOMY SEIKO, Tokyo). Afterward, 2 μl of 25 ng/ml trypsin (Promega) diluted in 50 mM NH₄HCO₃ was added to each gel piece and incubated for 30 min at 4°C, and then 30 μl of 50 mM NH₄HCO₃ was added and the spots were incubated overnight at 37°C. Supernatants were collected, peptides were further extracted twice with 0.1% trifluoroacetic acid (TFA) in 50% acetonitrile for 15 min, and the supernatants were then pooled. Peptide extracts were vacuum-dried and dissolved with 2 μl of 50% acetonitrile and 0.1% TFA, and then aliquots of 0.5 μl were applied on the target and air-dried. Subsequently, 0.5 μl of matrix solution (CHCA saturated in 50% acetonitrile and 0.1% TFA) was added to the dried samples and allowed to dry again.

Samples on the MALDI target plates were then analyzed by ABI 4800 Proteomics Analyzer MALDI-TOF/TOF mass spectrometer (Applied Biosystems). A total of 800 shots were accumulated for MS analyses. MS/MS analyses were performed using air, at collision energy of 2 KV. MASCOT search engine (version 2.1, Matrix Science) was used to search all of the tandem mass spectra. GPS Explorer™ software (version 3.6.2, Applied Biosystems) was used to create and search files with the MASCOT search for peptide and protein identification. Protein identities were obtained using Mascot searching engine against SwissProt non-redundant sequence databases selected for “Mus musculus” taxonomy.

Swiss-Prot database was searched with accession numbers of all the identified proteins. Much more protein information could be found, such as, theoretical molecular weight (MW), theoretical protein isoelectric point (pl), and subcellular location, et al. Moreover, protein interaction prediction was performed with String software for differentially expressed proteins.

**Bioinformatics analysis**

Western blot was used to validate the differential abundance of mass spectrometry identified proteins. Aliquots of 20 μg of proteins were diluted in Laemmli sample buffer (final concentrations: 50 mM Tris, pH 6.8, 10% glycerol, 2% SDS, 0.01% bromophenol blue) and separated by SDS-PAGE following standard procedures. After electrophoretic transfer of proteins onto 0.45 μm PVDF membranes (Millipore), the membranes were blocked with 5% w/v skimmed milk in TBST (50 mM Tris, pH 8.0, 150 mM NaCl, and 0.1% Tween-20) for 1 h. Membranes were then incubated in primary antibody solution in TBST containing 0.02% sodium azide for 2 h. Membranes were washed 3 times in TBST (5 min each time), and then incubated with the appropriate horseradish peroxidase-coupled secondary antibody for 1 h (Santa Cruz, USA). After washing 3 times with TBST, the reaction was detected by chemiluminescence with ECL reagents (Pierce Biotechnology, USA). All membranes were exposed and scanned by Carestream Image Station 4000R (Carestream Health, USA). A semi quantitative analysis based on OD was performed using Quantity One software (Bio-Rad, USA).

**Statistical analysis**

Statistical features in DeCyder were used for the evaluation of the DIGE gels. For all protein spots, comparison between SAKI and control groups was calculated, as change in volume ratios and with Student t-test as selection criteria. Protein spots differentially expressed between groups (filtering conditions: at least 50% change of ratios between groups and t-test P<0.05) were extracted. For western blot analyses, two-tailed non-paired Student’s t-test was performed.
Phosphorylated MYL12B as a potential plasma biomarker for SAKI

Results

Differentially expressed kidney proteins between SAKI and control mice

According to the DeCyder software analysis, about 2600 protein spots were constantly detected in each gel and quantified, normalized, and inter-gel-matched. A total of 4 obvious protein spots were up-regulated between SAKI and the controls. The differentially expressed protein figures showed greater than 1.5-fold change in the expression level were shown in Figure 1 (P<0.05).

Identification of differentially expressed proteins by MALDI-TOF/TOF MS

A total of 4 proteins have been successfully identified. The up-regulated proteins included actin (ACTB), myosin regulatory light chain 12B (MYL12B), myosin regulatory light polypeptide 9 (MYL9), and myosin regulatory light chain 12A (MYL12A). The detailed information of these proteins was listed in Table 1.

Bioinformatics analysis

As shown in Table 1, the protein MW and pl were listed. All the proteins except MYL9 had the extracellular location. Interestingly, all the four up-regulated proteins had the interaction relationships directly Figure 2.

Validation of MYL12B by western blotting with kidney samples

To validate the proteomics results and further evaluate the nature and importance of some of the identified proteins, mono-dimensional (1D) Western blot analyses were performed. Among all the varied proteins, MYL12B was validated by western blot. Interestingly, as shown in Figure 3, there was no change of kidney tissue proteins between the SAKI group and control group, however, phosphorylated MYL12B was detected to be consistent with the proteomics data.

Exploration of MYL12B by western blotting with plasma samples

In order to check whether the phosphorylated MYL12B could be the plasma biomarker for SAKI, the plasma samples were performed by Western blotting. As shown in Figure 4, phosphorylated MYL12B was found similarly to be increased in SAKI plasma, while MYL12B was changeless in plasma of control group.

<table>
<thead>
<tr>
<th>Spot No.</th>
<th>Protein name</th>
<th>Gene name</th>
<th>Accession No.</th>
<th>MW(kDa)/pl</th>
<th>Ion score</th>
<th>Fold change</th>
<th>Extracellular location</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Actin, cytoplasmic 1</td>
<td>Actb</td>
<td>P60710</td>
<td>42.1/5.29</td>
<td>131</td>
<td>2.56</td>
<td>Yes</td>
</tr>
<tr>
<td>2</td>
<td>Myosin regulatory light chain 12B</td>
<td>Myl12b</td>
<td>Q3THE2</td>
<td>19.8/4.71</td>
<td>162</td>
<td>1.64</td>
<td>Yes</td>
</tr>
<tr>
<td>3</td>
<td>Myosin regulatory light polypeptide 9</td>
<td>Myl9</td>
<td>Q9CQ19</td>
<td>19.9/4.8</td>
<td>90</td>
<td>1.58</td>
<td>No</td>
</tr>
<tr>
<td>4</td>
<td>Myosin regulatory light chain 12A</td>
<td>Myl12a</td>
<td>D3YV37</td>
<td>12.3/5.1</td>
<td>86</td>
<td>1.52</td>
<td>Yes</td>
</tr>
</tbody>
</table>

*Spot No. is the unique sample spot protein number that refers to the labels in Figure 1; *Swiss-Prot database accession number; *Theoretical molecular weight (kDa) and theoretical protein isoelectric point; *Ion score of more than 66 is significant (P<0.05); *Fold change of the differentially expressed renal proteins in SAKI mice.

Figure 2. Protein interaction net for varied proteins.
Discussion

Sepsis is a leading cause of acute kidney injury in clinical practice. The diagnosis of SAKI requires the diagnosis of sepsis and subsequent occurrence of acute kidney injury [13]. AKI occurs frequently in critically ill patients with sepsis, in whom it doubles the mortality rate and half of the survivors suffer permanent kidney damage or chronic kidney disease [16, 17]. To date, studies of the pathobiology of SAKI have focused on several aspects, including alterations in global renal blood flow (RBF), intrarenal hemodynamics, renal bioenergetics, and immune-mediated renal injury [18]. Although several investigational therapies have shown promise; however, most require further verification in high-quality trials to establish their efficacy and safety in patients with SAKI.

Fortunately, the application of innovative technologies such as functional genomics and proteomics to human and animal models of kidney disease has uncovered several novel candidates that are emerging as biomarkers and therapeutic targets [19]. Numerous novel bio-

Figure 3. Western blotting analysis of MYL12B with kidney samples between SAKI and control Group. A. Protein bands detected with MYL12B and phosphorylated MYL12B antibodies by Western Blotting. B. The relative quantification from WB was shown in the bar graph, error bar revealed standard deviation. The two-tailed non-paired Student’s t-test was performed using GraphPad Prism 5.0 (P<0.05).

Figure 4. Western blotting analysis of MYL12B with plasma samples between SAKI and control group. A. Protein bands detected with MYL12B and phosphorylated MYL12B antibodies by Western Blotting. B. The relative quantification from WB was shown in the bar graph, error bar revealed standard deviation. The two-tailed non-paired Student’s t-test was performed using GraphPad Prism 5.0 (P<0.05).
markers have been found to be up-regulated in kidney injury, among which cystatin C and NGAL are the most studied. In the management of SAKI, early goal directed therapy may be potentially useful, but requires further validation in large clinical trials [13]. An ideal biomarker of SAKI with highly sensitivity and specificity has not been identified yet.

In the present study, we firstly used 2D-DIGE combined MALDI-TOF/TOF MS to identify kidney biomarkers of SAKI. 4 up-regulated protein spots corresponding to 4 unique proteins were identified. These proteins have not been reported as biomarkers for SAKI in previous studies. After the bioinformatics analysis, 3 of them had extracellular location, so we supposed that they might be excreted in to blood, which is idea body fluid for discovering biomarkers. Furthermore, all of them had interaction relationships, we deducted that they might form one protein complex to fulfill some special functions in the kidney cells of SAKI.

Myosin II is an actin-binding protein composed of MHC (myosin heavy chain) IIls, RLCs (regulatory light chains) and ELCs (essential light chains) [20]. Myosin II expressed in non-muscle tissues plays a central role in cell adhesion, migration and division [21]. In non-muscle cells, the action of myosin II is controlled by RLCs, which contains three highly conserved non-muscle RLCs named MYL12A, MYL12B and MYL9. In particular, RLCs undergo phosphorylation at the Ser19 and Thr18 sites, which increases the Mg2+-ATPase activity of MHC in the presence of actin [20]. Some research revealed that MYL12A and MYL12B are crucial for maintenance of the stability of MYH9, MYH10 and MYL6, which leads to normal cell actomyosin function [20].

According our results, among all the up-regulated proteins, there are three highly conserved non-muscle RLCs of the mouse: MYL12A, MYL12B and MYL9 (MYL12A/12B/9). After using MYL12B antibody to check the protein expression level in renal tissue, no positive changes were seen, appearing not parallel to the DIGE results. So we changed the phosphorylated MYL12B antibody to check, surprisingly, phosphorylation level of MYL12B was increased at least two times in SAKI kidney tissue comparing with control group. Therefore, We indicated that phosphorylation of these RLCs may be played a central role to maintain the stability of myosin II and cellular integrity, which was very important to keep functional balance for renal cells.

Furthermore, many genes are up-regulated in the damaged kidney with the corresponding protein products appearing in plasma and urine. So we supposed that some kidney proteins might be excreted in to blood, which is idea body fluid for discovering biomarkers. As a result, phosphorylation level of MYL12B in plasma was consistent with the proteomics data. We speculated that phosphorylated MYL12B might be located in extracellular exosomes, which can bring some important information for communicating between blood and kidney. Hence, phosphorylated MYL12B maybe as one of the potential plasma biomarkers for early prediction of SAKI.

In conclusion, the quantitative proteomics analysis is a valuable tool for biomarker screening of SAKI. Phosphorylated MYL12B may be a putative biomarker that may be associated with the progression and development of SAKI. The potential of utilizing this marker for screening and treating SAKI warrants further investigations.

Acknowledgements

This work was supported by National Natural Science Foundation of China (No. 81101467, 81071529), and Natural Science Fund of Guangdong Province (No. 101510101002000001).

Disclosure of conflict of interest

None.

Address correspondence to: Dr. Lei Su, Department of Intensive Care Unit, General Hospital of Guangzhou Military Command, Guangzhou 510010, Guangdong, China. Tel: 86-20-88653484, Fax: 86-20-88653484; E-mail: suleigz@hotmail.com

References


