Identification of Hub Genes Associated with the Development of Acute Kidney Injury by Weighted Gene Co-Expression Network Analysis

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Keywords

Weighted gene co-expression network analysis · Acute kidney injury · Rplp1 · Lgals1

Abstract

Background: Acute kidney injury (AKI) is a severe clinical syndrome, causing a profound medical and socioeconomic burden worldwide. This study aimed to explore underlying molecular targets related to the progression of AKI. **Methods:** A public database originated from the NCBI GEO database (serial number: GSE121190) and a well-established and unbiased method of weighted gene co-expression network analysis (WGCNA) to identify hub genes and potential pathways were used. Furthermore, the unbiased hub genes were validated in 2 classic models of AKI in a rodent model: chemically established AKI by cisplatin- and ischemia reperfusion-induced AKI. **Results:** A total of 17 modules were finally obtained by the unbiased method of WGCNA, where the genes in turquoise module displayed strong correlation with the development of AKI. In addition, the results of gene ontology revealed that the genes in turquoise module were involved in renal injury and renal fibrosis. Thus, the hub genes were further validated by experimental methods and primarily obtained Rplp1 and Lgals1 as key candidate genes

related to the progression of AKI by the advantage of quantitative PCR, Western blotting, and in situ tissue fluorescence. Importantly, the expression of Rplp1 and Lgals1 at the protein level showed positive correlation with renal function, including serum Cr and BUN. **Conclusions:** By the advantage of unbiased bioinformatic method and consequent experimental verification, this study lays the foundation basis for the pathogenesis and therapeutic agent development of AKI.

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Introduction

Acute kidney injury (AKI) is a common clinical syndrome, including a variety of pathophysiological processes [1, 2]. Actually, the recovery of renal function in AKI is often incomplete and gradually develops into CKD, which eventually evolves into ESRD [3]. AKI is a pivotal contributor of CKD and consequent ESRD [4]. In the progression of AKI transitions into CKD, structural abnormalities are manifested as matrix expansion depositing in both mesangium and tubulointerstitium. Targeting

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Table 1. The primers used for qRT-PCR

Gene name	Primer sequence (5'→3')	
Lgals1	F:AACCTGGGGAATGTCTCAAAGT	R:GGTGATGCACACCTCTGTGA
Col3a1	F:CTGTAACATGGAACTGGGGAAA	R:CCATAGCTGAACTGAAAACCACC
Col6a2	F:GCTCCTGATTGGGGGACTCT	R:CCAACACGAAATACACGTTGAC
Rplp1	F:CTCGCTTGCATCTACTCCGC	R:AGAAAGGTTTCGACGCTGACAC
Mgp	F:GGCAACCCTGTGCTACGAAT	R:CCTGGACTCTCTTTGGGCTTTA

on renal fibrosis after AKI has been considered to be a therapeutic approach to minimize the transitions [5–7].

Elevated plasma Cr is considered to be a classic criterion for diagnosing AKI in the past decades. But it may not be a proper biomarker since it often suggests the balance of production and excretion of Cr [8]. Although eliminating the short- and long-term outcomes of AKI is urgent, therapeutic therapies and early detection biomarkers are still limited. In addition, there are presently no clinically valid biomarkers for the diagnosis of renal fibrosis after AKI. Hence, it is beneficial to develop more therapeutic therapies and figure out the potential biomarkers for the treatment of fibrosis and delaying the progression [1, 8].

Due to flawed methodologies of molecular biology, there are still certain limitations in the comprehensive exploration of the entire biological system in diseases [9]. As a systematic biological method for describing the genetic association patterns between different samples, weighted gene co-expression network analysis (WGCNA) is mainly used to screen and identify highly collaborative modules. According to the internality of the module and the relationship between modules and phenotypes, it has been widely used in the study of omics analysis to mine potential biomarkers or therapeutic targets [10–12]. Compared with data that only focus on differential expression, WGCNA has the following advantages: it can take full advantage of information, associate interesting alternations of phenotypes, and avoid the defects of differential expression analysis artificially setting thresholds. What is more, WGCNA can not only analyze the mRNA level of samples but also be used for micro-RNA and proteomics research, which is of great help in finding potential biological markers and therapeutic targets [13, 14].

In the present study, we performed a well-established bioinformatic method – WGCNA – to analyze the expression profile (NCBI GEO serial number: GSE121190) and aimed to identify the potential pathways and genes

associated with the progression of AKI. Importantly, we used experimental methods to further verify the hub genes, so as to provide new insights for the occurrence, development, and treatment of AKI.

Materials and Methods

Data Preprocessing

The data set was obtained from the GEO database (<https://www.ncbi.nlm.nih.gov/geo/>). In this study, 12 samples of GSE121190 data set were used for WGCNA analysis. Twelve samples included control group and folic acid-induced 3-/7-/14-day AKI group (3 samples per group). The R package “limma” is used for background correction and normalization processing with the original data. If multiple probes match with 1 gene, the average value is calculated as the gene expression value [9, 15].

Co-Expression Network Construction and Functional Analysis

Then, the soft threshold is calculated by WGCNA algorithm. The scale-independent value and average connectivity of modules under different soft thresholds were tested by the gradient method in order to determine the appropriate soft threshold [13, 16, 17]. By selecting appropriate soft threshold values, the co-expression network was constructed to ensure the authenticity of the results, and the minimum number of genes in each module was not <30. Then, use the R package “Clusterprofiler” toolkit to perform GO analysis [17–19].

Verification of Hub Gene

Next, we analyzed the hub genes according to the threshold value in each module and visualized using Cytoscape software. The hub genes were treated as the following criterion: (a) absolute value of gene significance (GS), FA 3/7/14 days ≥ 0.2 , and (b) the genes in the main module were analyzed by the STRING database (<http://www.string-db.org/>) to generate the protein-protein interaction (PPI) network, and the top 5 genes of highest connectivity were treated as hub genes for further verification [20].

Cisplatin- and Hypoxia-Ischemia-Induced Animal Models

Male C57BL/6 mice (SPF level, 6~8-week-old, 20~25 g) were purchased from Chengdu Dashuo Experimental Animal Co., Ltd. (SCXK [Sichuan] 2015-030) and housed and bred under standard conditions of care at the Experimental Animal Center of Southwest Medical University (SYXK [Sichuan] 2018-065). The mice

Fig. 1. Construction of collaborative expression module. **A** Clustering dendrogram of 12 samples. **B** Analysis of network topology for various soft thresholding (power). **C** Co-expression module constructed by dynamic hybrid algorithm. Every leaf node represents a gene, and the different colors represent different modules in the tree diagram.

were classified into 3 groups ($n = 6$ per group): control, Cis-AKI, and IRI-AKI; mice in the Cis-AKI group were injected cisplatin (Sigma-Aldrich, USA) (15 mg/kg) by intraperitoneal injection [21]. Mice in the IRI group were anesthetized with 1% pentobarbital sodium and placed on a small thermostatic electric blanket to keep body temperature at 36°C. After anesthesia, bilateral flanking

incisions were taken, and the arteriovenous arteries were clamped with arterial clips for 35 min. Saline was added dropwise to keep the kidneys moist every 5 min. After the arterial clip was removed, the incisions were sutured, and the mice were placed in 37°C environment [22, 23]. At the end of the experimental period, the mice were killed at days 3 and 7, respectively. Mouse blood samples were

Table 2. Number of genes in 17 co-expression modules

Modules	Freq
Light cyan	71
Midnight blue	75
Cyan	84
Salmon	85
Tan	86
Green yellow	94
Magenta	99
Purple	99
Pink	106
Red	130
Black	130
Green	206
Yellow	297
Brown	421
Gray	524
Blue	1,219
Turquoise	1,620

obtained before the cervical dislocation. All experimental procedures were approved by the Ethics Committee of Southwest Medical University (approval number: 201812-55).

Histological Analysis and Detection of Renal Function

The kidney was made into paraffin sections according to conventional methods. Briefly, the kidney tissues were fixed with 10% formaldehyde, dehydrated, and embedded in paraffin [24]. The sections were dehydrated and stained with hematoxylin and eosin (Beyotime, China). Ultimately, the sections were photographed with a virtual slide microscope (VS120-S6-W, Olympus, Japan). At the same time, serum Cr and BUN were measured according to the kit instructions (Nanjing JianCheng, China).

RNA Isolation and Quantitative PCR

Total RNA was extracted using an RNA extraction kit (Tiangen, Beijing) and complementary DNA was obtained using the Reverse Transcription Kit (Promega, Shanghai, China) according to the manufacturer's instructions. Then, quantitative PCR was performed in LightCycler[®] 480 II Real-Time PCR System (Roche, Germany). Primers were synthesized by Sangon Biotech (Shanghai, China), and sequences are available in Table 1.

Western Blot

Total proteins were isolated and dissolved in RIPA buffer. Next, protein concentrations were detected using a BCA Protein Assay Kit (Beyotime Biotechnology, China). The protein samples were run on 12% sodium dodecyl sulfate-polyacrylamide gel and transferred to PVDF membranes (Millipore, Danvers, MA, USA). Membranes were blocked with 5% bovine serum albumin at room temperature for 1 h and incubated at 4°C overnight with the indicated primary antibodies: anti-Lgals1 (Cell Signaling Technology, Danvers, MA, USA, 1:1,000) and anti-Rplp1 antibodies (Invitrogen, Waltham, MA, USA, 1:500). Afterward, PVDF membranes were washed with Tris-buffered Saline Tween and incubated at

Fig. 2. The fitting curve in 3 main modules. **A–C** Averaged expression levels of 30 genes within blue, brown, and turquoise modules, respectively, at all time points.

room temperature for 1 h with a secondary antibody (Invitrogen, Waltham, MA, USA; 1:10,000). Bands were visualized using the ECL Chemiluminescence Kit (Thermo, Waltham, MA, USA) and analyzed using ImageJ software.

In situ Immunofluorescence

For tissue immunofluorescence, kidney tissues were fixed with 10% formaldehyde solution and dehydrated, respectively, with 10, 20, and 30% sucrose, followed by embedding in OCT. The samples were then made into 4-μm sections and washed 3 times with PBS for 5 min. The tissues were blocked with 5% BSA for 1 h at room temperature and then incubated at 4°C overnight with the indicated primary antibodies: anti-Lgals1 (Cell Signaling Technology, Danvers, MA, USA; 1:200) and anti-Rplp1 antibodies (Invitrogen, Waltham, MA, USA; 1:100). The next day, tissues were washed with PBS for 5 min 3 times, followed by incubation with Alexa Fluor[®] 488-conjugated secondary antibodies (Cell Signaling Technology, Danvers, MA, USA; 1:200) and at room temperature for 1 h. The images were captured by a virtual slide microscope (VS120-S6-W, Olympus, Japan).

Statistical Analysis

Real-time PCR and Western blot statistical analyses were performed by GraphPad Prism 7.0 (GraphPad Software, La Jolla, CA, USA). One-way ANOVA was used if more than 2 comparison groups exist. $p < 0.05$ was considered as a statistical difference.

In the next step, we converted the adjacency matrix to a topological overlap matrix to reduce noise and false correlations. A system clustering tree was established through topological overlap matrix, as shown in Figure 1C. And the number of genes in each module was set as no less than 30 by the dynamic hybrid shear algorithm. Finally, 17 modules were obtained from the system cluster tree, and the number of genes contained in each co-expression module is shown in Table 2.

Key Modules and Hub Gene Screening

Afterward, we analyzed the top 3 modules (brown, blue, and turquoise modules) and sought to find out the hub genes. The expression patterns of genes in the 3 modules were analyzed. As presented in Figure 2, the result revealed that blue and brown modules are negatively correlated with AKI progression, whereas the turquoise module is positively correlated with AKI progression. We then performed the functional analysis in each module and found that the genes contained in the brown, blue, and turquoise modules are strictly related to the kidney, genitourinary system development, some essential metabolic processes, and extracellular matrix (ECM) deposition, which are strictly related to renal fibrosis; in particular, the turquoise module is thought to play an essential role in the development of renal fibrosis after AKI (details presented in Fig. 3A and see online suppl. Fig. 1A, 2A; see www.karger.com/doi/10.1159/000511661 for all online suppl. material). We then sought to find hub genes in each module, and after preliminarily filtering, the top 5 genes – Lgals1, Rplp1, Col3a1, Col6a2, and Mgp – in the turquoise module were treated as hub genes (as shown in Fig. 3B). We also performed a similar analysis in the blue and brown modules but failed to find hub genes considering their expression patterns and GS (as presented in online suppl. Fig. 1B, 2B).

Evaluation of Histological Pathology and Renal Function

Next, we constructed Cis-AKI and IRI-AKI animal models. As shown in online suppl. Figure 3a, HE staining results suggested that tubular necrosis existed in Cis-AKI/IRI-AKI models, and the injury accentuated over time. Meanwhile, we could also observe that compared with the control group, a rapid increase in serum Cr and BUN in a time-dependent manner. Collectively, all these results indicated the successful construction of AKI animal models.

Verification of Hub Gene

To further confirm the reliability of the WGCNA method and screen hub genes closely related to the progression of renal fibrosis, on the above basis, we aimed to validate the expression of top 5 hub genes in Cis-AKI and IRI-AKI animal models by advantage of real-time PCR, Western blotting, and in situ immunofluorescence. The mRNA expression levels of Lgals1, Rplp1, Col3a1, Col6a2, and Mgp in the kidney of AKI mice were detected by real-time PCR. There are many research reports indicated that Col3a1, Col6a2, and Mgp are related to the process of renal fibrosis [23, 25, 26]. However, for Lgals1 and Rplp1, no studies have been done on renal fibrosis after AKI, so Western blot and in situ immunofluorescence were used to further verify the expression of Lgals1 and Rplp1. As shown in Figure 4B, compared with the normal group, the expression levels of Lgals1 and Rplp1 in Cis-AKI and IRI-AKI mice were also significantly increased. Furthermore, we evaluated the location of Lgals1 and Rplp1 by in situ immunofluorescence. As shown in Figure 5, Rplp1 is mainly located in the glomeruli and renal tubular, and Lgals1 distributes in the interstitial and renal tubular. Importantly, we also validated the correlation between Rplp1/Lgals1 and BUN/Cr, and the results indicated that Rplp1/Lgals1 at the protein level were positively correlated with BUN/Cr (all $R > 0.5$, $p < 0.05$) (Fig. 4D–E).

Discussion

Increasing evidence suggests that AKI will eventually develop into CKD/ESRD, with a higher incidence [4, 27]. So far, there are limited therapies on the transition from AKI to CKD [28–30]. Hence, a better understanding of the pathophysiological mechanism and identification of potential biomarkers are greatly helpful to prevent this transition and improve its prognosis. In this study, we performed WGCNA to analyze microarray data related to the transition from AKI to CKD. A total of 17 modules were obtained, and the main 3 gene modules were employed for further analysis. The results from this study suggested that the 3 gene modules are closely related to

Fig. 4. Validation of the hub genes in the turquoise module. **A** Quantification of the mRNA expression of Lgals1, Col3a1, Rplp1, Mgp, and Col6a2 by Q-PCR. **B** Western blot was employed to investigate the indicated protein expression. **C** Quantification of protein expression by scanning densitometry. *** $p < 0.001$ compared with the control group. **D, E** The correlation between Rplp1/Lgals1 and BUN/Cr (all $R > 0.5$, $p < 0.05$).

(For figure see next page.)

Fig. 5. Validation of the location of the hub genes in the turquoise module. **A, B** The location and expression of Lgals1 and Rplp1 by in situ immunofluorescence, respectively.

the development of renal fibrosis after AKI in GO classification, for example, renal system development, renal vascular development [31, 32], some metabolic processes [33, 34], mitochondrial inner membrane, organelle inner membrane, and ECM [35–37]. ECM imbalance is a major cause of renal fibrosis. After AKI, fibrosis is affected by multiple factors such as accumulated inflammatory cells, activated myofibroblasts, and so on [30, 33]. Therefore, the present study focused on the turquoise module as the module is tightly related to the ECM.

Next, we filtered the genes in the turquoise module by calculating the GS, followed by generating the PPI network. From the PPI network in the turquoise module, it can be concluded that 6 genes S100a6, Col3a1, Col6a2, Mgp, Rplp1, and Lgals1 displayed the highest connectivity with other neighboring genes and located in the center of the PPI network. S100a6 is encoded by being a member of the S100 protein family. It has been reported in the literature that S100a6 may regulate cell proliferation and participate in immune-mediated pathological processes

of renal epithelial-mesenchymal transition and fibrosis [38, 39]. Col3a1 and Col6a2 are collagen type III alpha-1 and collagen type VI alpha-2 chains, respectively. They encode collagen, bind to extracellular machinery proteins, and are extremely important in cell-matrix components. When fibrosis occurs in organs such as liver and kidney, the expression of these collagens is elevated, which is an essential indicator for judging the progress of the disease and the observation of the therapeutic effect [23, 25]. Mgp, a matrix of Gla protein, is a member of the osteocalcin/Mgp family that is expressed after CKD and is associated with renal prognosis [26, 40]. In line with previous studies, our research also revealed that S100a6, Col3a1, Col6a2, and Mgp are potential early diagnostic markers of renal fibrosis after AKI.

Rplp1, a gene encoding a ribosomal protein that is part of the 60S subunit, plays an important role in the elongation step of protein synthesis. Rplp1 is highly expressed in multiple human cancer cells and its lacking will affect protein synthesis, leading to apoptosis [41, 42]. Lgals1, also known as Galectin-1, is a member of the β -galactoside-binding protein family and plays a role in regulating apoptosis, cell proliferation, and cell differentiation. Lgals1 is a research hot spot in the field of tumors, and many recent studies have reported that it also plays a vital role in the process of organ fibrosis. It has been indicated that Lgals1 is highly expressed in kidneys of type I and type II diabetes and is a new marker of renal fibrosis [43–45]. However, it is worth noting that Rplp1 and Lgals1 have not been reported in renal fibrosis after AKI. In the present study, we have preliminary proved that Rplp1 and Lgals1 might be used as potential biomarkers in renal fibrosis after AKI by bioinformatics and experimental manner. Our multiple experimental analyses indicated that Lgals1 and Rplp1 display dramatically increased in the kidneys of IRI-AKI and Cis-AKI, and the expression of the 2 genes is increased after 3 days and decreased after 7 days, which is consistent with the results analyzed by WGCNA bioinformatics method.

It should be noted that there are several limitations of the study: first, the number of samples used for analysis is relatively insufficient which may omit or decrease the accuracy of analysis. Second, the hub genes were further investigated only in the AKI rodent model rather than human specimens which may also hamper the translation of the findings from the mouse to human. There are many studies on LGALS1 in human cancer, and its expression is elevated in kidney cancer. Targeting LGALS1 can inhibit the development of its tumors. Although RPLP1 has been found in endometriosis, gynecological tumors, and

other diseases, it has not been studied in human kidneys. Further investigations of hub genes on human specimens such as urinary excretion may better elucidate their biological role clinically. In conclusion, the present study laid the foundation for further studying the role of Rplp1 and Lgals1 in AKI.

Conclusions

In summary, this study identified key genes related to AKI through WGCNA analysis, and experimentally found 2 hub genes with potential for further research, Rplp1 and Lgals1. However, the specific mechanism in the pathogenesis of AKI remains further research. The selected critical candidate genes provide new research directions for the prevention, diagnosis, treatment, and prognosis of renal fibrosis.

Statement of Ethics

All animals were treated in accordance with the National Institutes of Health Guidelines for use and care of research animals.

Conflict of Interest Statement

The authors declared that there are no conflicts of interest.

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Author Contributions

Li Wang and Jianchun Li conceived and coordinated the study. Xiao Lin wrote the paper. Jianchun Li revised the paper. Jianchun Li carried out the bioinformatics analysis. Xiao Lin performed the animal experiment and carried out the real-time PCR, IHC, and IF. Xia Zhong and Jieke Yang carried out the WB and collected the mouse renal samples. Ruizhi Tan performed statistical analysis. All authors reviewed the results and approved the final version of the manuscript.

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