Identification of biomarkers, immune infiltration landscape, and treatment targets of ischemia–reperfusion acute kidney injury at an early stage by bioinformatics methods

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Abstract

Background: Mechanisms underlying ischemia/reperfusion injury-acute kidney injury (IRI-AKI) are not fully elucidated. We conducted an integrative analysis of IRI-AKI by bioinformatics methods.

Methods: We screened gene expression profiles of the IRI-AKI at early phase from the Gene Expression Omnibus (GEO) database. Differentially expressed genes (DEGs) were identified and enrichment pathways were conducted based on gene ontology (GO), Kyoto Encyclopedia of Genes and Genomes (KEGG) database, and Gene set enrichment analysis (GSEA). Immune cell infiltration analysis was performed to reveal the change of the microenvironment cell types. We constructed protein–protein interaction (PPI), and Cytoscape with plug-ins to find hub genes and modules. We performed robust rank aggregation (RRA) to combine DEGs and analyzed the target genes for miRNA/transcription factor (TF) and drug-gene interaction networks.

Results: A total of 239 and 384 DEGs were identified in GSE87024 and GSE34351 separately, with the 73 common DEGs. Enrichment analysis revealed that the significant pathways involve mitogen-activated protein kinase (MAPK) signaling, interleukin-17, and tumor necrosis factor (TNF) signaling pathway, etc. RRA analysis detected a total of 27 common DEGs. Immune cell infiltration analysis showed the plasma cells reduced and T cells increased in IRI-AKI. We identified JUN, ATF3, FOS, EGR1, HMOX1, DDIT3, JUNB, NFKBIZ, PPP1R15A, CXCL1, ATF4, and HSPA1B as hub genes. The target genes interacted with 23 miRNAs and 116 drugs or molecular compounds such as curcumin, staurosporine, and deferoxamine.

Conclusion: Our study first focused on the early IRI-AKI adopting RRA analysis to combine DEGs in different datasets. We identified significant biomarkers and crucial pathways involved in IRI-AKI and first construct the immune landscape and detected the potential therapeutic targets of the IRI-AKI by drug-gene network.

Keywords: Ischemia/reperfusion injury, Acute kidney injury, Robust rank aggregation, Immune infiltration, Drug-gene interaction network

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Background

Acute kidney injury (AKI), characterized by a rapid decrease in glomerular filtration rate, is a universal disease in hospital with high morbidity and mortality. It is reported that the incidence of AKI is 10–15% of all hospitalizations [1] and approximately 50% in the intensive care unit.
care unit [2]. The ischemia–reperfusion injury (IRI) is the most common cause of AKI [3], which often occurs after surgery and contributes to adverse outcomes in kidney transplantation. The mismatch between supply and demand of oxygen is the central pathophysiology of the IRI/AKI leading to oxidative metabolism reduction and further injury of tubular epithelial cells [4]. Though several biomarkers, such as kidney injury molecule-1 (KIM-1), neutrophil gelatinase-associated lipocalin (NGAL), and interleukin-18 (IL-18), have been studied (KIM-1), neutrophil gelatinase-associated lipocalin (NGAL), and interleukin-18 (IL-18), have been studied, no one can substitute the creatinine in the clinical setting since low specificity to predict and diagnose AKI as early as possible and reducing the heterogeneity of different datasets, we chose the two datasets (GSE87024 and GSE34351 published in high-level journals without being analyzed well before (Table 1). For GSE87024, we extracted the IRI-6 h (GSM2319037, GSM2319038, GSM2319039) and sham group (GSM2319034, GSM2319035, GSM2319036) and for GSE34351, we chose the IRI-4 h (n = 3, GSM847661, GSM847662, GSM847663) and control group of wild-type mice (n = 3, GSM847664, GSM847665, GSM847666). The method of performing the IRI-AKI model in the dataset GSE34351 was clipping the left renal for either 16 min or 23 min after right nephrectomy, which was like the dataset GSE87024 making the left renal occluded for 17.5 min.

The platform for GSE87024 is GPL6887, Illumina MouseWG-6 v2.0 expression beadchip, while GSE34351 is GPL1261 [Mouse430_2], Affymetrix Mouse Genome 430 2.0 Array. Normalization of these data was carried out with the "limma" R package.

Identification of differentially expressed genes
We applied the linear model and empirical Bayes model analysis by the "limma" R package to find the DEGs and calculate the differential expression. The [log2fold change (FC)] > 1.5 and p-value < 0.05 were used as the significant criteria. Heatmaps and volcano plots of DEGs were conducted using the "Pheatmap" and "ggplot2" packages in R 4.0.0. An unsupervised principal component analysis (PCA) method was performed to extract two features from each group. The overlapping DEGs were further visualized by the "VennDiagram" R package.

Functional and pathway enrichment analysis
We conducted the Gene ontology (GO) terms and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway enrichment analysis of DEGs in different datasets. GO analysis can find the biological characteristics in the biological process (BP) of the genes. KEGG analysis offers a comprehensive knowledge of bio-interpretation of cellular processes and identifies shared pathways of co-expressed genes. We completed and visualized the
Table 1 Characteristics of the individual studies

| GEO ID   | Platform | Published Time | Organism      | Strain     | Tissue Type | IRI time | Sample size | Citation (PMID) | Citation (Journal) |
|----------|----------|----------------|---------------|------------|-------------|----------|-------------|------------------|--------------------|
| GSE87024 | GPL6887  | 2016           | Mus musculus  | C57BL/6    | IRI vs Sham kidney | 6 h      | 3 vs 3      | 26,823,548       | JASN               |
| GSE34351 | GPL1261  | 2012           | Mus musculus  | C57BL/10   | IRI vs Sham kidney | 4 h      | 3 vs 3      | 22,895,517       | KI                 |

GEO: Gene Expression Omnibus, IRI: Ischemic renal injury
JASN: Journal of American Society of Nephrology, KI: Kidney International
analysis by the "ClusterProiler" V3.16.1 package [11] (significant criteria is \( p < 0.05 \) and q-value < 0.05) and "DOSE" v3.16.0 [12].

**Gene set enrichment analysis (GSEA) of the two expression data sets**

GSEA is a powerful analytical method to identify whole gene sets, which share common chromosomal location, biological function, or regulation by comparison with knowledge-based databases accumulating large-scale expression data sets [13]. We conducted the GSEA of the two datasets in GO and KEGG respectively. Each analysis performed 1000 times of arrangement of the gene set. The criteria of the significantly enriched gene sets were \( P\)-value < 0.05. The GSEA analysis was performed by the "ClusterProiler" V3.16.1 package.

**Evaluation of immune cell infiltration**

To evaluate the immune cell role and change in the IRI-AKI, we conducted the immune cell infiltration analysis by CIBERSORT method [14]. CIBERSORT can accurately estimate the immune composition of tissue. We conducted this analysis by "CIBERSORT.R" script and visualized the results by "pheatmap" and "ggpubr" R packages.

**Construction and analysis of protein and protein interactions (PPI) network**

We searched the common DEGs in the online tool STRING (http://www.string-db.org/) to construct the PPI network showing interactions between genes or proteins. We conducted the confidence score of 0.4 as the cut-off value, visualized the PPI network of DEGs by Cytoscape software [15]. Cytoshubba and CytoNCA [16] plug-in were employed to identify the hub genes separately. We adopted 11 methods (MCC displays a satisfying comparative performance) in Cytohubba and 3 evaluation indexes (degree centrality (DC), betweenness centrality (BC), closeness centrality (CC) applied in the CytoNCA. We further extracted hub modules using another plug-in - Molecular Complex Detection (MCODE) with the cut-off score of 2.

**Robust rank aggregation (RRA) method to find the DEGs**

We used the de-bach effect, the robust rank aggregation, and probabilistic models to integrate sorted lists of different gene expression profiles from the different protocols or measurement platforms. Based on each gene freely arranged in each data assumption, we scored the rank vector by the order-based statistical analysis and defined the final score of each vector as the minimum \( p\)-value. The \( p\)-value is corrected to determine whether the ranking of a specific gene is statistically significant, and multiple checks to assess the stability of the acquired \( p\) values. We repeated leave-one-out cross-validation 10,000 times and calculated the averaged \( p\) values from all rounds. If a gene ranks high in the results of all DEGs, the smaller \( p\)-value was by the RRA method and with the greater probability of the DEGs' robustness. This process was conducted by the “RobustRankAggreg” R package [17].

**Transcription factor (TF)-gene interactions and gene-miRNA network of the combined DEGs and hub genes**

We constructed the gene-miRNA network and TF-gene interactions of the combined DEGs from RRA analysis and hub genes in the NetworkAnalyst [18] (https://www.workanalyst.ca/), which is a comprehensive web platform for gene expression analysis. The gene-miRNA network is based on the miRTarBase (http://mirtarbase.mbc.nctu.edu.tw/php/download.php), while TF-gene interactions based on the ENCODE (http://cistrome.org/ BETA/).

**Construction of Drug Gene Interaction network**

The Drug Gene Interaction Database (DGIdb) version 3.0.2 (https://www.dgidb.org) consolidates, synthesis, and normalizes drug-gene interactions and gene druggability information from 30 disparate sources [19]. We searched the DEGs from the RRA analysis and hub genes in the DGIdb to explore potential drugs or molecular compounds that interacted with the genes. The drug-gene interaction network was visualized by the Cytoscape software.

**Results**

**Identification of differentially expressed genes**

After standardization of the two datasets (Figure S1), 239 (187 up-regulated and 52 down-regulated genes) and 384 DEGs (259 up-regulated and 125 down-regulated genes) were extracted from the GSE87024 and GSE34351. PCA score trajectory plots indicated that the IRI and CON groups didn’t overlap suggesting the apparent differences between the two groups (Fig. 2 A-B). Heatmaps showed the DEGs could discriminate between the IRI and control (CON) groups (Fig. 2 C-D). The volcano plots visualized the distribution of DEGs (Fig. 2E-F).

**Functional and pathway enrichment analysis**

For up-regulated genes in GSE87024, the KEGG pathway analysis acquired the 23 significant pathways with the top 3 pathways are ‘TNF signaling pathway, MAPK signaling pathway, and IL-17 signaling pathway (Fig. 3A). Cneplot visualized the conjunction between genes and the enrichment pathway(Fig. 3B). GO functional enrichment analysis showed up-regulated genes were mainly involved in GO terms about the regulation of vasculature development,
response to extracellular stimulus, and intrinsic apoptotic signaling pathway (Fig. 3C). GO Cluster plot showed the interaction between clusters and genes in GO terms (Fig. 3D). Relationships of different GO terms were visualized in GPlot (Fig. 3E). The down-regulated genes weren't enriched in any pathways.

For GSE34351, up-regulated genes were enriched in 28 significant pathways, including MAPK, IL-17, TNF, and Estrogen signaling pathways (Fig. 4A). Emaplot displayed
the interaction of enriched pathways, and Cneplot visualized the interaction between genes and the enrichment pathways (Fig. 4B-C). Significant enrichment of GO terms included the unfolded protein, regulation of vasculature development, transcription from RNA polymerase II promoter to stress, and topologically incorrect protein (Fig. 4D). GO circle and cluster plot showed the distributions of the genes and GO terms (Fig. 4E-F). No pathway was enriched in the down-regulated genes. Venn diagram showed the common 73 DEGs from the two expressional datasets (Fig. 5A).

Combined DEGs enrichment analysis of GO and KEGG showed the significant pathways involved C−type lectin receptor, NF−kappa B, and GnRH signaling pathways (Table S1-2, Fig. 5B-D).

RRA to find the combined DEGs
A total of 25 significant up-regulated genes and two down-regulated genes were identified in the RRA analysis. The heat map showed the expression profile of the top 20 most significant up and down-regulated genes. Each square represented a different gene, and each column represented the expression level of all genes in a data set (Fig. 10).

TF-gene interactions and gene-miRNA network
Gene-miRNA network showed both Adm and Jun modulated by 5 miRNAs, while Egr1 regulated by 3 miRNAs (Fig. 11A). The top 3 targeted DEGs for TFs were Junb, 2410006H16Rik, and Nfkbiz modulated by 26, 18, and 16 TFs separately (Fig. 11B).

Construction of drug gene interaction network
The drug-gene interaction network indicated that JUN, DDIT3, CFRT, FOS, ADM, interact with 44, 26, 22, 10, and 7 drugs or molecular compounds separately. The deferoxamine, glutamine, sirolimus, indomethacin are connected with JUN and DDIT3. (Table S3, Fig. 12).

Discussion
In this study, we screened two expression profiles with the same tissue and similar genetic background mice in early-stage IRI-AKI from the GEO database, adopted RRA analysis to integrate the DEGs for detecting the potential biomarkers and pathways in the pathogenesis of IRI-AKI. We identified the MAPK, TNF, and IL-17 signaling pathways in KEGG database. Regulating the vasculature development, responding to extracellular stimulus, and intrinsic apoptotic signaling pathway were identified...
Fig. 3 (See legend on previous page.)
in GO database. Further GSEA analysis showed the PI3K-Akt signaling pathway, cytokine-cytokine receptor, positive regulation of cellular component biogenesis, and reproductive structure development pathway are crucial in IRI-AKI. Combined with the analysis by Cytohubba and CytoNCA, we figured out the JUN, ATF3, FOS,
Fig. 5  Visualization of the enrichment of common differentially expressed genes (DEGs) from two datasets. A Venn diagram presents a combination of all differentially expressed genes of two datasets. B Bar chart shows the significant KEGG pathways enriched by the 73 DEGs. C Bubble chart displays the significant GO terms. D GO plot shows the interacts with different enriched GO terms.
Fig. 6  Visualization of the gene set enrichment analysis (GSEA) of the GSE87024.  
A GSEA plots shows the most enriched gene sets in KEGG of all detected genes in the GSE87024.  
B The top 10 most significant up-regulated enriched gene sets in KEGG.  
E The top terms enriched in GO database.  
D The top 10 most significantly enriched terms in GO database.
EGR1, HMOX1, DDIT3, JUNB, NFKBIZ, PPP1R15A, CXCL1, ATF4, and HSPA1B as hub genes. GO, KEGG and GSEA analysis showed that the MAPK, TNF and IL-17 signaling pathways are crucial in IRI-AKI development. MAPK signaling pathway consists of four branches, namely ERK, JNK, p38, and ERK5. Activation of p38 and JNK signaling is a feature of acute kidney disease. The relative levels of JNK, p38, and ERK activation have been considered to determine cell fate after kidney damage. Selective inhibitors of p38 MAPK seemed
to be effective in rodent models of acute kidney disease [20]. Remote ischemic pretreatment plays a role in preventing IRI from developing through activating JNK, p38, and MAPK kinase [21]. Several drugs or molecular compounds mitigate IRI via the MAPK pathway [22, 23]. TNF, considered as a crucial mediator in cell proliferation, cell death, and differentiation, interacts with two cell surface receptors: TNFR1 and TNFR2 (TNFRs) [24]. Studies showed the level of circulating TNF was increased during IRI-AKI causing renal cell damage via neutrophil-mediated inflammatory injury and apoptosis [25]. IRI mice with genetic deletion of TNFR1 displayed a significant lessening in renal injury and inflammation [26]. Pretreatment soluble TNFR2 fusion protein to neutralize TNF-

\[ \text{A} \]

\[ \text{B} \]

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\text{Fig. 8 Evaluation and visualization of immune cell infiltration. A Heatmaps of the immune cell abundance in GSE87024; B Heatmaps of the immune cell abundance in GSE34351; C Barplot of the proportion of the immune cell in each sample (GSE87024); D Barplot of the proportion of the immune cell in each sample (GSE34351); E Box plot of the immune cell distribution in different group (GSE87024); F Box plot of the immune cell distribution in different group (GSE34351)\]
biomarkers (Cxcl1 and Jun, Fos, Nfkbiz, Hmox1) were researched in other types of AKI, 2 genes (Atf4 and Egr1) play role in I/R injury of other organs and three genes (Ppp1r15a, Hspa1b, and Ddit3) had not been reported in AKI or IRI researches. Atf3 could protect against IRI-AKI via suppressing p53 and inducing p21. In vitro studies showed it attenuated cell apoptosis by interacting with Nicotiflorin [30, 31]. CXCL1-CXCR2 signaling axis played an important role in alleviating cisplatin-induced AKI by regulation of inflammatory response [32]. Jun was studied in acute kidney injury including aristolochic acid-induced AKI, crush syndrome induced AKI, and myoglobinuric AKI but not IRI-AKI [33–35]. Inhibitor of c-Fos/activator protein-1 could decrease the production of TNF-α and other downstream molecules, which protect against LPS-AKI [36]. FosB induced the elevated expression of matrix metalloproteinase-2 in the cardiac IRI mice [37]. Studies found the NF-κB/miR-376b/NFKBIZ

Fig. 9 Outcomes of Protein–protein interaction (PPI) network. A PPI network of the common 73 differentially expressed genes (DEGs) of the 2 datasets. Node color reflects the grade of Degree scores calculated in the CytoNCA. (Green color represents a higher degree, and yellow color represents a lower degree). B 10 hub genes identified from the Cytohubba. C, D, E Subnetwork of hub modules acquired in the MCODE with MCC scores are 6, 4.8, and 4.5 respectively.
negative feedback loop adjusted intrarenal inflammation and alleviated renal damage in septic AKI [38]. HMOX1 long GT tandem repeats are associated with the occurrence of AKI in sickle cell anemia people [39]. Atf4 was related to endoplasmic reticulum stress, amino acid starvation, mitochondrial stress, and oxidative stress. It was reported that MIF-2/D-DT increased proximal tubular cell regeneration via ATF4-dependent pathways in IRI mice [40]. Egr1 was mainly studied in myocardial IRI and it may serve as a major regulator of remote preconditioning [41]. For Ppp1r15a, Hspa1b, and Ddit3, we haven’t found any related AKI or IRI studies, which should be further verified in experimental studies.

We first conducted the drug-gene interaction network to identify the potential targets of IRI - AKI. Our results showed that staurosporine is a common molecular compounds interacting with CXCL2 and DDIT3. Considered as a protein kinase C inhibitor, staurosporine could protect against the impairment of working memory in IRI gerbils and rats [44, 45]. Curcumin interacts with both DDIT3 and cystic fibrosis transmembrane conductance regulator (CFTR). It is a diketone compound extracted from the plant turmeric. Some animal studies have shown that curcumin can protect the I/R injury and toxin-induced injury [46, 47]. Nowadays, researchers have designed a stepwise-targeting chitosan oligosaccharide conjugate, which can convey curcumin to renal tubular epithelial cells and remove excessive reactive oxygen species (ROS), to treat acute kidney injury [48]. Crofelemer, an inhibitor of the CFTR, was applied to alleviate pain in women with irritable bowel syndrome-diarrhea (IBS-D) as well as treat noninfectious diarrhea in HIV-positive patients receiving antiretroviral therapy [49]. Further studies should be conducted to discover the

![Fig. 10](image-url) Heatmap of the Robust Rank Aggregated showed the top 20 differentially expressed genes aggregated of the two datasets. The red represents log FoldChange (FC) > 0, while green represents log FC < 0. The value in the box displays the log FC value.
Fig. 11 The networks of target gene-miRNA and TF-gene interactions. **A** Target gene-miRNA network. The pink circle nodes are the genes, and the blue octagon nodes are the miRNAs. **B** TF-gene interactions network. The red circle nodes are the genes, and blue octagon nodes are the transcription factors (TFs).
roles of the drugs or molecular compounds as potential therapeutic targets.

Our study have some strengths. First, we screened all the datasets about the IRI-AKI in GEO and focused on the early onset of this disease selecting the mice with similar genetic background to reduce individual differences. Second, we applied multiple bioinformatic methods to identify common DEGs that are potentially involved in the disease. In our limited knowledge, this was the first study applied the RRA analysis, a robust and compelling approach to integrate different datasets on IRI-AKI. Third, we performed the GSEA method utilizing all genetic expression information in datasets to find the crucial pathways in IRI-AKI. Different methods are applied to detect the hub genes and hub modules. Forth, we further analyzed the target genes for miRNA/TF. Fifth, we analyzed the signature of the immune cell in and found the T cell increasing in IRI-AKI. Last, we first conducted the drug-gene interaction network and identified 116 drugs or compounds as potential therapeutic targets of IRI-AKI giving new insights for further study.

There were some limitations in our study. First, to aggregate samples with similar genetic background mice and IRI-AKI occurrence time, we only selected two datasets and extracted a total of 12 samples. Though different times of IRI in GSE87024, we chose the earliest time after IRI-AKI to analyze. Since the limited sample numbers, we can’t apply the weighted gene co-expression network analysis (WGCNA) to construct gene co-expression networks in our study. Second, we focused on the microarray and didn’t include the RNA-seq, so we lack the data of miRNA and lncRNA. However, we constructed the TF-gene interactions and gene-miRNA network utilizing the open database. Third, the DEGs acquired from the RRA analysis are limited, we didn’t perform further GO and KEGG pathway analysis. Fourth, we didn’t validate
the hub genes identified in this study in AKI patients or experiment, which is a part of our future work.

Conclusions
To conclude, our study identified 10 hub genes and 3 modules, key pathways involved in early IRI-AKI diagnosis and treatment utilizing various bioinformatic methods. We constructed the immune landscape and provided new insights and implications for further experimental confirmation.

Abbreviations
AKI: Acute kidney injury; ATF3: Activating Transcription Factor 3; ATF4: Activating Transcription Factor 4; CON: Control; CXCL1: C Motif Chemokine Ligand 1; DDI3: DNA Damage Inducible Transcript 3; DEGs: Differentially expressed genes; DGIdb: Drug Gene Interaction Database; EGR1: Early Growth Response 1; GO: Gene ontology; GSEA: Gene set enrichment analysis; HMOX1: Heme Oxygenase 1; HSPA1B: Heat Shock Protein Family A Member 1B; IBS-D: Irritable bowel syndrome-diarrhea; IL-17: Interleukin-17; IL-18: Interleukin-18; IRI: Ischemia/reperfusion injury; KEGG: Kyoto Encyclopedia of Genes and Genomes; KIM-1: Kidney injury molecule-1; MAPK: Mitogen-activated protein kinase; MCODE: Molecular Complex Detection; NGAL: Neutrophil gelatinase-Associated Lipocalin; NFKBIZ: NF-kappa-B Inhibitor zeta; PCA: Principal Kinase; PPP1R15A: Protein phosphatase 1 Regulatory Subunit 15A; ROS: Reactive oxygen species; RRA: Robust rank aggregation; TF: Transcription factor; TNF: Tumor necrosis factor; WGCNA: Weighted gene co-expression network analysis.

Supplementary Information
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Author’s contributions
RL You conceived and designed the study, performed the study, analyzed the data, wrote the paper, prepared figures and/or tables. ZG HeYang, YX Ma performed the study, Hua Zheng, JF Lin, PL Ji analyzed data; LM Chen conceived and designed the study, wrote the paper, reviewed drafts of the paper. The author(s) read and approved the final manuscript.

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Availability of data and materials
All data generated or analysed during this study are included in this published article and its Supplementary information files.

Declarations

Ethics approval and consent to participate
Not applicable.

Consent for publication
Not applicable.

Competing interests
The authors declare that they have no competing interests.

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