1898

Original Paper

Bioinformatic Analyses of Renal Ischaemia-Reperfusion Injury Models: Identification of Key Genes Involved in the Development of Kidney Disease

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Key Words

Abstract

Background/Aims: To develop a novel strategy for the treatment of kidney disease, we explored potential molecular targets involved in the development of renal ischaemia-reperfusion injury (IRI). ***Methods:*** The Gene expression profile data of GSE27274, including controls and rats subjected to renal IRI and reperfusion for 24 h (IR24) or 120 h (IR120), was obtained from the Gene Expression Omnibus database. Differentially expressed genes (DEGs) were analysed using the limma package. Gene Ontology (GO) and pathway functional enrichment analyses of common DEGs were carried out. Protein-protein interactions (PPI) and miRNA-DEG network analyses were performed using the STRING database and WebGestalt, respectively, followed by network construction using Cytoscape. ***Results:*** In total, 80 common DEGs (41 up- and 39 downregulated genes) between IR24 and IR120 were screened. Genes encoding tissue inhibitor of matrix metalloproteinase-1 (*Timp1*), secreted phosphoprotein 1 (*Spp1*) and dimethylglycine dehydrogenase (*Dmgdh*) were identified as hub genes in the PPI network and may be significant in the development of renal IRI. Upregulated *Spp1* was enriched in the inflammatory response, and downregulated *Dmgdh* was enriched in the catabolic process of the amino acid betaine. In reactome pathway analyses, *Spp1* was enriched in toll-like receptor signalling, and *Dmgdh* was enriched in glycine, serine and threonine metabolic pathways. The common DEGs were mainly regulated by 15 miRNA clusters. ***Conclusion:*** *Timp1*, *Spp1*, *Dmgdh*, miR-142-5p and miR-181a may be potential targets or biomarkers for the development of renal IRI.

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1899

Zhu et al.: Bioinformatic Analyses of Renal Ischemia-Reperfusion Injury Models

Introduction

Acute kidney injury (AKI) is associated with high healthcare costs, morbidity and mortality in the clinic and has become an important public health concern worldwide [1]. Recently, AKI has been suggested to lead to long-term outcomes, including the development and progression of chronic kidney disease (CKD) and end-stage renal disease (ESRD), even in those patients who recover [2, 3]. Although the needed to eliminate the short- and long-term outcomes of AKI is urgent, no effective therapy exists to prevent the development of these renal diseases.

Renal ischaemia-reperfusion injury (IRI) is one of the major causes of AKI in hospital settings [4]. The complicated cellular pathophysiology in IRI development, including cell adhesion, proliferation and cytokine secretion, is associated with inflammation, the innate immune response, and injury to tubular, endothelial and vascular components [5, 6]. Many factors are thought to relate to the development of renal IRI. Yu TM et al. [7] demonstrated that RANTES (regulated on activation, normal T-cell expressed and secreted), produced by renal tubular cells, is a major chemokine in the development of renal IRI. Furthermore, an inhibitor of the complement C1 fraction (C1INH) prevented inflammatory reactions, neutrophil migration and the development of fibrosis in a murine model of renal IRI [8]. In addition, microRNAs (miRNAs), non-coding RNAs that regulate their target mRNAs via a post-transcriptional repression process, have a potential effect in renal IRI development [9]. miR-21 is upregulated to protect against apoptosis of tubular epithelial cells during the development of renal IRI [10]. On the other hand, increased miR-24 promotes apoptosis of endothelial and tubular epithelial cells [11]. While multiple signalling pathways have been reported, the molecular mechanisms underlying the development of renal IRI are still unclear.

In the present study, the differentially expressed genes (DEGs) were screened from the GSE27274 dataset between sham controls and two groups of rats subjected to different time-points of reperfusion injury. By performing functional enrichment, protein-protein interaction (PPI) and miRNA-target gene network analyses, several candidate genes were demonstrated to be potential targets or biomarkers for diagnosing or treating the development of renal IRI.

Materials and Methods

Affymetrix microarray data acquisition

Gene expression profile data from GSE27274 were obtained from the NCBI Gene Expression Omnibus (GEO, http://www.ncbi.nlm.nih.gov/geo/) database. The dataset was based on the GPL6101 Illumina platform ratRef-12 v1.0 expression beadchip and deposited by Krishnamoorthy A et al. [12]. Included in the dataset were 24 samples from bilateral ischaemia-reperfusion (IR) kidney injury and its sham controls. Renal cortex and medulla samples obtained at 24 h or 120 h of reperfusion were used for further analysis. To clarify the genes and pathways in the whole kidney, cortex and medulla samples from the corresponding rats were pooled.

Data preprocessing and DEG analysis

The raw array data were normalized by the Robust Multiarray Average (RMA) method [13]. The mean value of multiple probes for a single gene was calculated. The linear models for microarray data (limma) package [14] in R were used to analyse the DEGs from renal IRI compared with those from sham controls, and the |log Fold Change (FC)| >1.5 and p-values < 0.05 were considered the threshold as previously described [15].

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1900

Zhu et al.: Bioinformatic Analyses of Renal Ischemia-Reperfusion Injury Models

Gene ontology (GO) and pathway functional enrichment analyses

The online functional annotation tool DAVID (Database for Annotation, Visualization and Integrated Discovery) was used to understand the biological meaning of genes [16]. The up- and downregulated DEGs in renal IRI were used to perform GO biological process (BP), cellular components (CC), molecular function (MF) and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway enrichment analyses using the DAVID functional classification tool. The cut-off values for the GO analysis were set at $p < 0.05$ and enrichment score more than 2 and for the KEGG Pathway analysis at $p < 0.05$.

PPI network analysis

The Search Tool for the Retrieval of Interacting Genes (STRING) [17] database is a global resource for predicting PPIs. In the present study, DEGs were uploaded to the STRING online tool, and combined scores > 0.4 were selected as significant interactions. PPI networks were visualized by Cytoscape® version 3.4.0 software [18].

Interaction network analysis between DEGs and miRNAs

The WEB-based Gene Set Analysis Toolkit (WebGestalt) [19] is an online integrated data mining system. The regulatory miRNAs of both up- and downregulated DEGs were predicted by using WebGestalt, and the thresholds were set at p value < 0.05 and enriched genes > 2. Up- and downregulated DEGs and the miRNA network were visualized with Cytoscape.

Results

Identification of DEGs

The DEGs between sham controls and the samples obtained at 24 h (IR24) or 120 h (IR120) of reperfusion after renal ischaemia are shown in Fig. 1. Compared with the expression profiles from sham controls, a total of 504 DEGs (182 up- and 322 downregulated genes) were found in IR24 rats. Moreover, 111 DEGs (66 up- and 45 downregulated genes) were screened in IR120 rats. In total, 80 DEGs were common to both the IR24 and IR120 samples, with 41 upregulated and 39 downregulated genes (Fig. 2).
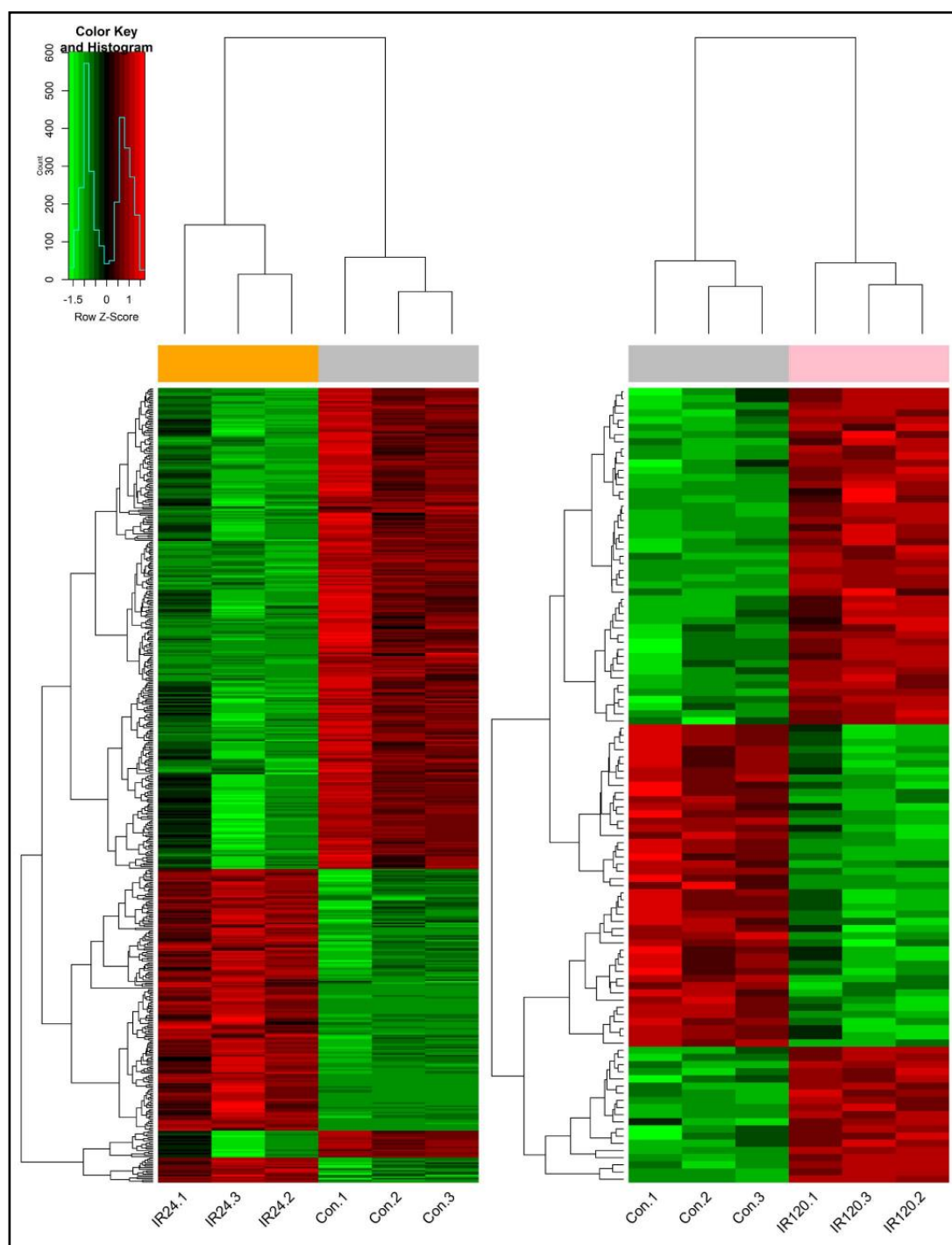
Functional enrichment analyses of common DEGs

To identify the functional enrichment in the development of renal IRI, GO enrichment analyses were performed for up- and downregulated common DEGs (Table 1). Upregulated common DEGs were significantly enriched in 27 GO terms for BP. The BP terms with the smallest P value in the two different reperfusion groups included inflammatory response, cell adhesion, innate immune response and cell proliferation. Downregulated common DEGs were enriched in amino-acid betaine catabolic processes, transmembrane transport, homocysteine and one-carbon metabolic processes.

Furthermore, significantly enriched pathways of up- and downregulated common DEGs were obtained by utilizing DAVID. As shown in Table 2, the main enriched KEGG pathways of upregulated DEGs were toll-like receptor signalling, complement and coagulation cascades and ECM-receptor interaction. Downregulated DEGs were enriched in glycine, serine, threonine, cysteine and methionine metabolic pathways.

PPI network

Based on the STRING database web tool, PPI networks of the 80 common DEGs were constructed with a combined score of >0.4. As shown in Fig. 3, the PPI had 36 nodes and 54 edges. Genes with the highest interaction degrees encoded tissue inhibitor of matrix metalloproteinase-1 (*Timp1*; degree=10), angiotensinogen (*Agt*; degree=8), dimethylglycine dehydrogenase (*Dmgdh*; degree=7) and secreted phosphoprotein 1 (also known as osteopontin; *Spp1*; degree=7).

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1901

Zhu et al.: Bioinformatic Analyses of Renal Ischemia-Reperfusion Injury Models

Fig. 1. The heat maps of the top 50 DEGs. The left panel represents DEGs of rats subjected to renal IRI and reperfusion for 24 h, the right represents reperfusion for 120 h. Each row represents a gene, and each column represents a sham control or a rat underwent renal IRI. Each colored square shows the relative change of a single gene in a single rat, red color indicates higher and green color indicates lower expression levels. Black color represents no change in expression.

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1902

Zhu et al.: Bioinformatic Analyses of Renal Ischemia-Reperfusion Injury Models

Fig. 2. Common DEGs in rats underwent renal IRI at two time-points of reperfusion. 24h and 120h indicate 20 min of ischemia followed by 24h and 120h of reperfusion, respectively. Venn diagram has shown 80 common DEGs at two groups of rats subjected to different time-points of reperfusion injury. The red and green arrows represent up- and downregulated genes, respectively.
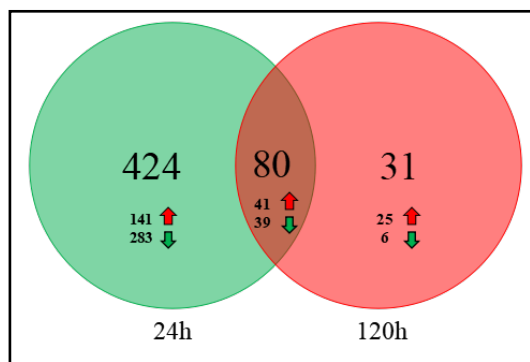
Table 1. The GO terms of the 80 common DEGs with P-Values < 0.05. BP: biological process; CC: cellular component; MF: molecular function; GO: gene ontology; Count: the DEGs number enriched in the corresponding GO term

GO ID	Term	Count	P-Value
Up-regulated genes			
	GO-BP terms		
GO:0071347	cellular response to interleukin-1	5	2.25E-05
GO:0006954	inflammatory response	6	1.36E-04
GO:0071222	cellular response to lipopolysaccharide	5	1.79E-04
GO:0009615	response to virus	4	3.65E-04
GO:0006898	receptor-mediated endocytosis	4	6.16E-04
GO:0007155	cell adhesion	5	9.69E-04
GO:0045087	innate immune response	5	9.69E-04
GO:0071354	cellular response to interleukin-6	3	1.04E-03
GO:0032496	response to lipopolysaccharide	5	1.26E-03
GO:0008284	positive regulation of cell proliferation	6	1.50E-03
	GO-CC terms		
GO:0005615	extracellular space	19	1.62E-13
GO:0070062	extracellular exosome	18	1.46E-07
GO:0009986	cell surface	10	4.66E-07
GO:0072562	blood microparticle	6	1.44E-06
GO:0005604	basement membrane	5	1.69E-05
GO:0031012	extracellular matrix	6	5.92E-05
GO:0031982	vesicle	5	1.44E-04
GO:0005578	proteinaceous extracellular matrix	5	8.01E-04
GO:0009897	external side of plasma membrane	5	9.78E-04
GO:0005576	extracellular region	7	1.04E-03
	GO-MF terms		
GO:0008201	heparin binding	5	7.30E-05
GO:0005125	cytokine activity	5	9.68E-05
GO:0002020	protease binding	4	8.82E-04
GO:0048248	CXCR3 chemokine receptor binding	2	7.78E-03
GO:0001618	virus receptor activity	2	2.32E-02
Down-regulated genes			
GO-BP terms			
GO:0006579	amino-acid betaine catabolic process	2	2.74E-03
GO:0009725	response to hormone	3	8.03E-03
GO:0050667	homocysteine metabolic process	2	1.23E-02
GO:0060351	cartilage development involved in endochondral bone morphogenesis	2	1.36E-02
GO:0051593	response to folic acid	2	2.57E-02
GO:0055085	transmembrane transport	3	3.61E-02
GO:0006730	one-carbon metabolic process	2	3.77E-02
	GO-CC terms		
GO:0070062	extracellular exosome	10	5.74E-03
	GO-MF terms		
GO:0072341	modified amino acid binding	2	1.00E-02

*Potential regulatory miRNAs associated with DEGs*

In AKI and CKD, microRNAs (miRNAs) are one of the most common epigenetic factors regulating gene expression [10, 20]. The online tool WebGestalt was used to retrieve potential regulatory miRNAs associated with DEGs, and the miRNA-DEG network was visualized with Cytoscape. As shown in Fig. 4A, the network of miRNAs and upregulated DEGs contained 17 nodes and 19 edges. *Adamts1, Hspa2, Fn1* and *Abcg1* had the highest degrees of 5, 3, 3 and

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1903

Zhu et al.: Bioinformatic Analyses of Renal Ischemia-Reperfusion Injury Models

Table 2. The Reactome pathway enrichment analyses of the 80 common DEGs (P-Values < 0.05)

Category	Term	Count	Genes	P Value
Up-regulated DEGs				
rno04620	Toll-like receptor signaling pathway	4	CXCL11, CD14, SPP1, CXCL10	1.34E-03
rno05150	Staphylococcus aureus infection	3	ICAM1, C1QB, CFI	6.74E-03
rno04610	Complement and coagulation cascades	3	C1QB, FGB, CFI	1.17E-02
rno04512	ECM-receptor interaction	3	LAMC2, SPP1, FN1	1.76E-02
rno05146	Amoebiasis	3	LAMC2, CD14, FN1	2.70E-02
Down-regulated DEGs				
rno01100	Metabolic pathways	11	MUT, MAT2A, CSAD, UPB1, BHMT, DMGDH, CYP2E1, CHPT1,	1.87E-04
rno00260	Glycine, serine and threonine metabolism	3	BHMT, DMGDH, CBS	3.56E-03
rno00270	Cysteine and methionine metabolism	3	MAT2A, BHMT, CBS	3.74E-03

Fig. 3. The PPI networks of DEGs. The red and green nodes represent the up- and downregulated DEGs, respectively. The predicated and precise results are indicated by black edges.
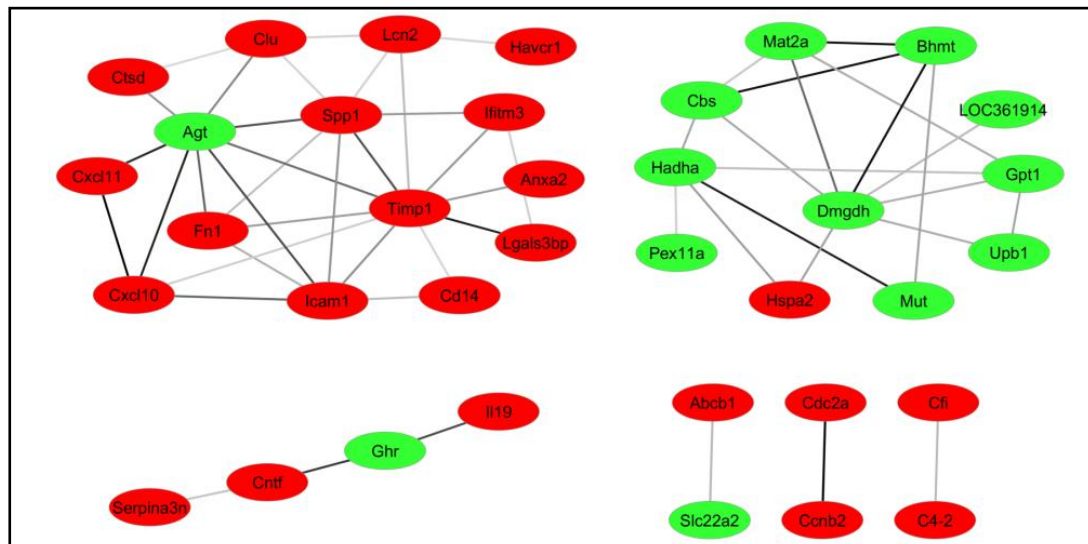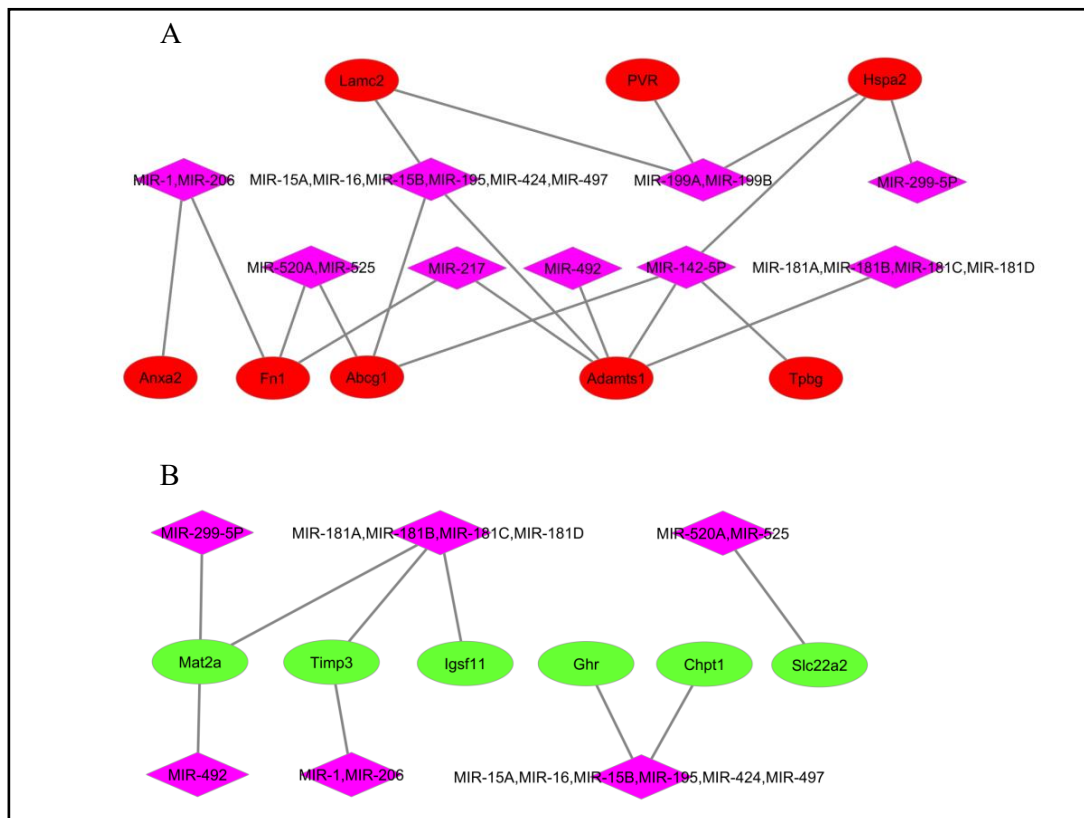

3, respectively. miR-142-5p was demonstrated to have an important role in regulating the highest number of upregulated DEGs, including *Hspa2, Abcg1, Adamts1* and *Tpbg*. On the other hand, the network of miRNA and downregulated DEGs contained 12 nodes and 9 edges (Fig. 4B). *Mat2a* and *Timp3* showed the highest degrees of 3 and 2, respectively. Moreover, miR-181a, miR-181b, miR-181c and miR-181d were found to regulate 3 of the target genes, *Mat2a, Timp3* and *lgsf11*.

Discussion

In the present study, 80 common DEGs were extracted at 24 h (IR24) and 120 h (IR120) of reperfusion after renal IRI in rats. *Spp1, Timp1* and *Dmgdh* demonstrated higher degrees in the PPI networks and may be potential key genes in the development of renal IRI. Interestingly, the upregulated DEGs were enriched in BPs and pathways associated with inflammation. On the other hand, the downregulated DEGs were enriched in the catabolic process and metabolic pathways of the amino-acid betaine. Finally, miR-181 was suggested to have a key role in renal IRI development.

Damaged renal cells secrete proinflammatory cytokines to promote inflammation, which in turn injures renal cells in AKI [1]. Inflammation has therefore been considered one of the most important mechanisms contributing to the pathophysiology of AKI, including renal IRI [21]. In this study, the upregulated DEG *Spp1* was a key gene in the PPI networks and was enriched in BP terms and pathways associated with inflammation. Spp1, also known as osteopontin, is a secreted glycoprotein that acts as a proinflammatory cytokine and is highly

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1904

Zhu et al.: Bioinformatic Analyses of Renal Ischemia-Reperfusion Injury Models

Fig. 4. Regulatory network of miRNA-common DEGs. Upregulated (A) and downregulated (B) networks are represented by miRNA (pink diamond), upregulated common DEGs (red circular nodes) and downregulated common DEGs (green circular nodes). Regulating relationships are represented by arrow edges.

expressed in the kidney [22]. Although Spp1 was demonstrated to prevent renal injury at 24 h after reperfusion of IRI in female mice [23], other investigators observed promotion of inflammation by Spp1 via enhanced infiltration of natural killer (NK) cells in IRI male mice [24, 25]. Furthermore, Spp1 also led to renal inflammation in chronic aldosterone-treated mice and in *ApoE*-deficient mice on a high-cholesterol diet [26, 27]. Although the role of Spp1 in the early stage of IRI is controversial depending upon sexual differences, it may at least have a potential role in promoting renal inflammation in males during IRI development.

Timp1 is the other hub gene identified by the PPI network analysis of upregulated DEGs. The protein encoded by *Timp1* is a member of tissue inhibitors of metalloproteinases (TIMPS), which are regulators of matrix metalloproteinases (MMPs) [28]. In addition to matrix degradation, TIMP1 also participates in inflammation, apoptosis and cell growth [29]. Although *Timp1* deficiency failed to ameliorate renal interstitial fibrosis in a murine model of unilateral urethral obstruction (UUO) due to compensatory increases in TIMP2, TIMP3 and PAI-1 mediating renal fibrosis [30], over-expression of TIMP1 deteriorated interstitial fibrosis by promoting the inflammatory response in the UUO model [31]. On the other hand, *Timp1* was also considered a urinary biomarker in cisplatin- and sepsis-induced AKI [32, 33]. Accordingly, TIMP1 may not be a direct target for the treatment of renal fibrosis, but it may become a potential biomarker for study of the development of renal IRI.

The third hub gene identified by the PPI network analysis of downregulated DEGs is *Dmgdh*, which was also enriched in BP terms related to the catabolic process and metabolic pathways of the amino-acid betaine. Dimethylglycine dehydrogenase, encoded by *Dmgdh*, is a mitochondrial matrix enzyme involved in the metabolism of choline to glycine [34]. Glycine was reported to possess anti-inflammatory and immunomodulatory roles against

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1905

Zhu et al.: Bioinformatic Analyses of Renal Ischemia-Reperfusion Injury Models

ischaemia-induced renal injury [35]. In fact, glycine protected against mild unilateral IRI (15 min ischaemia) [36]. Conversely, in severe bilateral IRI (40 min ischaemia) in rats, glycine exacerbated oxidative stress and renal cell injury through binding and activating N-methyl-D-aspartate (NMDA) receptors [37]. These two contrasting findings in glycine-treated rats may be attributed to sexual difference, strain background or degree of renal ischaemia. Overall, *Dmgdh* might at least play a role in mild IRI.

Up- and downregulated networks of miRNA-targeted DEGs were predicted and constructed. miR-142-5p in upregulated network was calculated to regulate *Adamts1*, *Hspa2*, *Abcg1* and *Tpbg*. In the downregulated network, miR-181a, miR-181b, miR-181c and miR-181d were predicted to regulate *Mat2a, Timp3* and *Igsf11*. Among these miRNAs, miR-142-5p was identified as a candidate biomarker of renal fibrosis [38]. miR-181a was documented to mediate cell apoptosis in cisplatin-treated HK-2 cells [39]. Furthermore, among these miRNA-targeted genes, *Timp3*-encoded protein has been demonstrated not only to protect against inflammation, apoptosis and tubulointerstitial fibrosis in the early stage of renal injury following UUO in mice [40] but also to inhibit breakdown enzymes of capillary basement membrane, including ADAMTS1 (a disintegrin and metalloproteinase with thrombospondin motif-1, *Adamts1*-encoded protein) [41]. ADAMTS1, a secreted metalloproteinase with angiogenesis inhibition, is considered as a mediator of renal vascular stability. Its expression was increased in proximal tubular cells following post-ischaemia of renal IRI and in pericytes after UUO administration [41, 42]. Therefore, miR-142-5p, miR-181a and their target genes may have critical roles in the development of renal IRI.

Although *Hmox1*, complement/coagulation cascades and the metabolic pathway of glycerophospholipid were previously reported to be potential targets for the early stage of renal IRI [43], our findings may provide novel targets or biomarkers for further research, diagnosis and treatment of renal IRI. Certainly, animal experiments and clinical studies are needed to confirm their significance in the development of renal IRI.

Conclusion

The present study pointed out that the *Spp1, Timp1,* and *Dmgdh* genes, with their associated BP terms and pathways, have potential roles in the development of renal IRI. Furthermore, miR-142-5p and miR-181a might be key miRNAs that modulate renal IRI development.

Acknowledgements

Disclosure Statement

The authors declare no competing interests.

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