Original Paper

Revealing the Underlying Mechanism of Ischemia Reperfusion Injury Using Bioinformatics Approach

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Key Words

Ischemia reperfusion injury • Differentially expressed genes • Protein-protein interaction network • MicroRNA-target gene network • Functional analysis

Abstract

Background/Aims: To reveal the potential pathogenesis of ischemia/reperfusion (I/R) injury. **Methods:** GSE9943 were downloaded from Genome Expression Omnibus database, including I/R and control samples for both Brown Norway (BN) and Sprague Dawley (SD) rats (3 rats/each group). Then differentially expressed genes (DEGs) were identified by limma package. miRNA-target gene network pairs were predicted using WebGestalt, and protein-protein interactions (PPI) were identified based on STRING database, followed by the networks construction using Cytoscape. Next, ClusterONE was used for modules screening. Furthermore, functional analyses were performed to common DEGs and genes. **Results:** Totally, 23 common DEGs of BR and SD rats were screened, enriched in functions, such as regulation of cellular protein metabolic process, response to wounding, proteinaceous extracellular matrix, and Enzyme inhibitor activity. MIR-29A, MIR-29B and MIR-29C were discovered both in up- and down-regulated miRNA-target gene networks. Genes in the PPI network were significantly disturbed in p53 signaling, complement and coagulation cascades pathway. Four modules were found significantly disturbed cytochrome P450, Serine/threonine protein kinase, calcium binding and Transient receptor potential channel protein domains. **Conclusion:** During I/R injury, many genes mutated, interrupting several biological functions, pathways and protein domains. MIR-29C and TRPC6 were suggested to be potential novel targets for this disease.

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Introduction

The first ischemia reperfusion injury (I/R) was recognized in 1970s and the term indicates tissue ischemia with inadequate oxygen supply followed by successful reperfusion

initiating a wide and complex array of inflammation responses [1]. Due to the severity of this disease, numbers of researchers have been engaging in revealing the potential pathogenesis. M Kawaguchi et al. discover that inflammation activation of cardiac fibroblasts is essential for myocardial ischemia/reperfusion injury [2]. Toll-like receptor-mediated signaling pathways was implicated in ischemia I/R [3]. Although several factors associated with I/R have been discovered, the mechanism of this disease is not fully indicated. Therefore, new targets needed to be discovered.

MicroRNAs (miRNAs) are integral elements in the post-transcriptional control of gene expression [4] and these small molecules have the potential to influence cellular behavior [5]. During last few decades, several miRNAs have been found playing roles in I/R development. For example: miRNA-320 is involved in the regulation of cardiac ischemia I/R by targeting heat-shock protein 20 [6]. miRNA-494 is down-regulated in murine I/R-injured and human infarcted hearts [7]. MiRNAs are regulators of target genes and miRNA-target gene pairs play important roles in biological behaviors. miR-124 is identified to be an endogenous regulator of Ku70 that improves I/R-induced brain injury [8]. Thus, in our study, the roles of miRNA-target gene pairs in I/R development were investigated.

Brown Norway (BN) rats strain was discovered to be dramatically resistant to cardiac and renal I/R injury compared with other strains, while Sprague Dawley (SD) rats are more susceptible with I/R injury [9]. Resistance to the development of tubular damage after renal ischemia was also observed in BN rats, and higher levels of heat-shock proteins and ablated oxidative stress was believed to be the putative mechanism of this resistance [10, 11]. BN rats strain is also reported to be an useful experimental model to investigate the mechanism of I/R injury compared with SD rats strain [12]. Despite of all the previously reported works, the genetic response of these two kinds of rats to ischemic insults was not clearly elucidated.

In the present research, the differentially expressed genes (DEGs) between I/R and control rats (BN and SD) were selected and their functions were analyzed. Additionally, the miRNA-target gene relationships and protein-protein interactions (PPIs) were predicted and the corresponding networks were constructed. Furthermore, the potential mechanism of I/R was revealed by clustering and functional analysis for PPI network. Based on the findings, the pathogenesis of I/R was investigated and several potential novel targets were discovered for this disease.

Materials and Methods

Data extracting and sampling

The data GSE9943 [13] were downloaded from the National Center for Biotechnology Information (NCBI) Genome Expression Omnibus (GEO, <http://www.ncbi.nlm.nih.gov/geo/>) database [14]. Totally 12 samples were available within the data, including two groups (I/R group and control group) for both Brown Norway (BN) and Sprague Dawley (SD) rats (n = 3 per group). Gene annotation information was also downloaded from the platform of GPL 2996 ABI Rat Genome Survey Microarray, which contained 26857 probes.

Data processing and DEGs identifying

The rows containing missing values were deleted from the original file, and then one file included 12925 rows and 12 columns was obtained. Next, limma package [15] in R (V.3.0.1) [16] was used to identify the DEGs between I/R group and control group in the two rat strains. Bayes testing [17] was applied to perform the multiple correction, and p values < 0.05 and |log fold change (FC)| > 1.0 were set as the threshold.

Functional enrichment analysis

DAVID (Database for Annotation, Visualization and Integrated Discovery [18]) was utilized to perform protein-domains, Gene Ontology (GO) [19] and pathway enrichment analyses based on hypergeometric analysis. The databases InterPro [20] and KEGG (kyoto encyclopedia of genes and genomes) [21] were used for protein-domains and pathway predication, respectively. P values less than 0.05 were set as the threshold.

MiRNA-target gene network

MicroRNA Target Analysis tool in WebGestalt (WEB-based GENE SeT Analysis Toolkit) [22] was used to predict the miRNAs for up- and down-regulated genes. miRNAs with multiple test adjustment p values (AdjP) less than 0.1 were selected. Then the miRNA-target gene network was constructed using Cytoscape [23].

Protein-protein interactions (PPI) network constructing

It has been discovered that functional links between proteins can be inferred from genomic associations between the genes that encode them, and the genes are often located in close proximity on the genome. STRING database (<http://www.bork.embl-heidelberg.de/STRING/>) [24] is a precomputed global resource for the exploration and analysis of these associations. STRING is updated continuously and currently, and now contains 261033 orthologs in 89 fully sequenced genomes. In the present study, STRING 9.0 was used to screen the interactions among the DEGs, and the PPIs with combined scores more than 0.4 were selected for PPI network construction. What's more, pathway annotation was conducted by DAVID with p value less than 0.05.

Module analysis of the PPI network

The Clustering analysis of the PPI network was performed using the ClusterONE in Cytoscape [25] with the minimum size as 5 and minimum density as 0.05, to select the functional modules. P values less than 1.0×10^{-5} were selected as threshold. Moreover, protein domain enrichment analysis of the modules was performed by DAVID and p values less than 0.05 were set as threshold.

Results

The identified DEGs

Using limma package, the DEGs between I/R and control BN and SD rats were identified. Finally, 92 DEGs were screened in BN rats, including 69 up-regulated and 23 down-regulated genes. Meanwhile, 176 DEGs were discovered in SD rats which contained 92 up-regulated and 84 down-regulated genes. Interestingly, 23 DEGs were found both in BN and SD rats, including up-regulated genes: Angpt14, Atp12a, Ccnb1, Emp3, Fenl, Foxml, and Gpnmb; down-regulated genes: Ces1, Cox8h, Gdf15, and Gsta5 (Figure 1).

Functional analysis of common DEGs

Further, GO analysis was performed to the 23 common DEGs and the significant terms with p values less than 0.05 were selected (Table 1). The common DEGs were discovered significantly enriched in 8 biological process related GO terms, including the regulation of cellular protein metabolic process ($p = 0.022$), response to wounding ($p = 0.023$), and M phase ($p = 0.027$). Totally 4 cellular component related terms were enriched, such as proteinaceous extracellular matrix ($p = 0.005$) and extracellular matrix ($p = 0.007$). Enzyme inhibitor activity was the only molecular function related term ($p = 0.039$).

MiRNA-target gene network

Based on the 245 DEGs between I/R and control rats, which included 142 up-regulated and 103 down-regulated genes, the corresponding miRNAs were predicted. Significant miRNA-target gene pairs were selected with AdjP < 0.1 and the miRNA-target gene network

Fig. 1. Identified DEGs between I/R and control rats. BN and SD represent Brown Norway and Sprague Dawley, respectively. Meanwhile, the red and blue arrows are up-regulated and down-regulated genes, respectively.

Table 1. The significant GO terms of the 23 common DEGs with P Values < 0.05

Category	Term	Description	Count	P Value
BP	GO:0032268	regulation of cellular protein metabolic process	4	0.022461188
BP	GO:0009611	response to wounding	4	0.023608951
BP	GO:0000279	M phase	3	0.027081944
BP	GO:0042060	wound healing	3	0.027081944
BP	GO:0007049	cell cycle	4	0.029170792
BP	GO:0040007	growth	3	0.039937324
BP	GO:0045861	negative regulation of proteolysis	2	0.040898315
BP	GO:0022403	cell cycle phase	3	0.046190442
CC	GO:0005578	proteinaceous extracellular matrix	4	0.004619661
CC	GO:0031012	extracellular matrix	4	0.006741095
CC	GO:0044421	extracellular region part	5	0.020594376
CC	GO:0005576	extracellular region	6	0.042916516
MF	GO:0004857	enzyme inhibitor activity	3	0.039374025

Note: BP, CC and MF are biological process, cellular component and molecular function, respectively. GO represents the gene ontology. Count is the DEGs enriched in the corresponding GO term. DEGs: differentially expressed genes.

was established by Cytoscape (Figure 2).

MiRNA-target gene network of up-regulated genes (Figure 2A), included 39 nodes and 77 edges. Genes, Dll1, Bdnf and Gmfb were discovered with the highest degree of 10, 9 and 9 respectively in this network. Meanwhile, MIR-29A, MIR-29B and MIR-29C were found regulated the most target genes (5), such as Slc16a1, Gmfb, Gpr37, Dnm3 and Fbn1.

MiRNA-target gene network of down-regulated genes contained 29 nodes and 35 edges. Igf1 and Nptx1 had the highest degrees of 7 and 4, respectively. Interestingly, the miRNAs which regulated most target genes were also MIR-29A, MIR-29B and MIR-29C. These three miRNAs regulated Igf1, Trim63, Gpr37 and Prepl in the network.

PPI network

The PPIs among 245 DEGs were predicted by SRTING and the significant ones with combined score > 0.4 were selected to construct the PPI network. The PPI is shown in Figure 3, containing 112 nodes and 228 edges. Genes which had the highest degrees included Agt (Degree = 16), Timp1 (Degree = 15), Egfr (Degree = 15), Cat (Degree = 15), and Top2a (Degree = 13).

Fig. 2. The miRNA-target gene network. A and B represent the up- and down-regulated network, respectively. The diamond nodes are miRNAs. The red and blue round nodes are up- and down-regulated target genes, respectively.

Fig. 3. The PPI network for DEGs between I/R and control rats. The nodes and edges represent the proteins (genes) and interactions, respectively. The thick edges stand for the large scores, indicating the predicted results are precise. Yellow node is the TRPC6 gene.

Pathway enrichment analysis for PPI network

Moreover, pathway enrichment analysis was conducted to the genes in the PPI network using DAVID and the significant pathways with p values < 0.05 were listed in Table 2. The most significant pathways were p53 signaling pathway [26, 27] ($p = 4.72\text{E-}04$) and complement and coagulation cascades [28] ($p = 6.20\text{E-}04$). Meanwhile, the DEGs enriched in these two pathways included CCNB1, CDC2, SERPINE1, A2M and FGG.

Module analysis

Four modules, Module 1 (Nodes = 35, Density = 0.119, Quality = 0.747, P -value = $1.019\text{E-}9$), Module 2 (Nodes = 14, Density = 0.615, Quality = 0.903, P -value = $2.459\text{E-}6$), Module 3 (Nodes = 12, Density = 0.530, Quality = 0.673, P -value = $5.715\text{E-}4$), and Module 4 (Nodes = 11, Density = 0.236, Quality = 0.565, P -value = $1.0\text{E-}3$) were obtained during PPI clustering analysis by using ClusterONE in Cytoscape and the result is shown in Figure 4.

Furthermore, we conducted protein domain enrichment analysis of the genes in the four Modules by DAVID, and the significant protein domains with p values < 0.05 are listed in Table 3. The protein domains in Module 1 significantly enriched in cytochrome P450 ($p = 1.19\text{E-}2$) and the genes included CYP4A1, CYP2D4V1, and CYP2D1. The protein domains in Module 2 significantly enriched in Serine/threonine protein kinase ($p = 7.84\text{E-}3$) and

Table 2. The significant pathways in PPI network with p values < 0.05

Term	Description	Count	Genes	P Value
rno04115	p53 signaling pathway	6	CCNB1, CDC2, SERPINE1, IGF1, CHEK1, CHEK2	4.72E-04
rno04610	Complement and coagulation cascades	6	A2M, FGG, FGB, SERPINE1, C4BPA, PLAUR	6.20E-04
rno04110	Cell cycle	7	CCNB1, PLK1, CDC2, CDC20, CHEK1, PTTG1, CHEK2	0.001502
rno04114	Oocyte meiosis	6	CCNB1, PLK1, CDC2, IGF1, CDC20, PTTG1	0.004815
rno00270	Cysteine and methionine metabolism	4	LDHC, LDHB, CTH, CDO1	0.004968
rno00982	Drug metabolism	5	GSTA3, ADH1, MAOB, CYP2D4V1, CYP2D1	0.00561
rno03450	Non-homologous end-joining	3	DCLRE1C, MRE11A, FEN1	0.007164
rno00640	Propanoate metabolism	3	LDHC, LDHB, PCCB	0.04269
rno04540	Gap junction	4	EGFR, CDC2, TUBB5, HTR2A	0.046936

Count is the DEGs enriched in the corresponding pathways.

Fig. 4. Modules in PPI network. There are 35, 14, 12, and 11 nodes in Module 1, 2, 3 and 4, respectively.

the genes included PLK1, CDC2, and CHEK1. The protein domains in Module 3 significantly enriched in calcium binding ($p = 1.48E-4$) and the genes included S100A4, S100A6, and S100A10. The protein domains in Module 4 significantly enriched in Transient receptor potential channel ($p = 1.48E-4$) and the genes included TRPC6, and TRPV6.

Table 3. The significant protein domains of genes in four modules with $p < 0.05$

Term	Description	Count	PValue	Genes
Module 1				
IPR002401	Cytochrome P450, E-class, group I	3	1.19E-02	CYP4A1, CYP2D4V1, CYP2D1
IPR017973	Cytochrome P450, C-terminal region	3	1.95E-02	CYP4A1, CYP2D4V1, CYP2D1
IPR008069	Cytochrome P450, E-class, group I, CYP2D-like	2	1.98E-02	CYP2D4V1, CYP2D1
IPR017972	Cytochrome P450, conserved site	3	2.01E-02	CYP4A1, CYP2D4V1, CYP2D1
IPR001128	Cytochrome P450	3	2.13E-02	CYP4A1, CYP2D4V1, CYP2D1
Module 2				
IPR002290	Serine/threonine protein kinase	3	7.84E-03	PLK1, CDC2, CHEK1
IPR008271	Serine/threonine protein kinase, active site	3	1.40E-02	PLK1, CDC2, CHEK1
IPR017442	Serine/threonine protein kinase-related	3	1.71E-02	PLK1, CDC2, CHEK1
IPR017441	Protein kinase, ATP binding site	3	2.27E-02	PLK1, CDC2, CHEK1
IPR000719	Protein kinase, core	3	2.89E-02	PLK1, CDC2, CHEK1
Module 3				
IPR013787	S100/CaBP-9k-type, calcium binding, subdomain	3	1.38E-04	S100A4, S100A6, S100A10
IPR001751	S100/CaBP-9k-type, calcium binding	3	1.48E-04	S100A4, S100A6, S100A10
IPR001464	Annexin	2	9.21E-03	ANXA1, ANXA2
IPR018252	Annexin repeat, conserved site	2	9.21E-03	ANXA1, ANXA2
IPR018502	Annexin repeat	2	9.21E-03	ANXA1, ANXA2
IPR001079	Galectin, carbohydrate recognition domain	2	9.86E-03	LGALS3, LGALS1
IPR011992	EF-Hand type	3	1.01E-02	S100A4, S100A6, S100A10
IPR013320	Concanavalin A-like lectin/glucanase, subgroup	2	4.97E-02	LGALS3, LGALS1
Module 4				
IPR004729	Transient receptor potential channel	2	6.59E-03	TRPC6, TRPV6
IPR012269	Aquaporin	2	6.59E-03	AQP3, AQP2
IPR000425	Major intrinsic protein	2	8.97E-03	AQP3, AQP2

Count is the DEGs enriched in the corresponding protein domains.

Discussion

In the present research, 23 common DEGs both in BR and SD rats were identified and these genes were found significantly interrupted several functions related with biological processes, cellular component and molecular function, including regulation of cellular protein metabolic process, response to wounding, M phase and wound healing. In addition, based on miRNA-target gene network, MIR-29A, MIR-29B and MIR-29C were discovered played important roles in I/R. Moreover, Agt, Timp1, Egfr, Cat, Cdc2, and Top2a were found to have the highest degrees in the PPI network and the genes mainly enriched in p53 signaling pathway and complement and coagulation cascades pathway. Finally, four modules in the PPI network were screened out, and they were significantly enriched in protein domains of cytochrome P450, Serine/threonine protein kinase, calcium binding and Transient receptor potential channel, respectively.

The relationships between miRNAs and target genes were predicted and two miRNA-target gene networks, including up- and down-regulated networks were constructed. Both in these two networks, MIR-29A, MIR-29B and MIR-29C were selected as they had the highest degrees, indicating these miRNAs may play important roles in disease development. Ouyang, Y.B., et al have reported that astrocyte-enriched miR-29a targets PUMA and reduces neuronal vulnerability to forebrain ischemia [29]. Additionally, Mateo Ziu et al. have found that expression of miR-29b is significantly decreased in neurons exposed to insulin-like

growth factor 1, a well documented neuroprotectant in ischemic models [30]. Our results are consistent with these previous findings. On the other hand, miR-29c was also discovered in our study but with no previous evidence. S Sengupta et al. have shown that miR-29c is expressed at one-fifth the levels in tumors as in normal epithelium [31]. Other researchers discover that miR-29c targets TNFAIP3, inhibits cell proliferation and induces apoptosis in hepatitis B virus-related hepatocellular carcinoma [32]. In our study, miR-29c was found connected with five target genes in up- and down-regulated networks, suggesting this may be a novel target for I/R research.

The established PPI network included 112 nodes and 228 edges. Angiotensinogen (Agt), tissue inhibitor of metalloproteinases-1 (Timp1), the epidermal growth factor receptor (Egfr), chloramphenicol acetyltransferase (Cat), (Csc2), and topoisomerase IIa (Top2a) were discovered to be connected with more than 12 genes in the network, indicating these genes may be involved in the disease developing. Further, these 112 nodes in PPI network were significantly enriched in p53 signaling pathway and Complement and coagulation cascades pathways. The p53 tumor-suppressor plays a critical role in the prevention of human cancer, and in response to various types of stress p53 becomes activated, cells can undergo marked phenotypic changes, ranging from increased DNA repair to senescence and apoptosis [33]. Thus, in our findings, genes may interrupt these two pathways to induce I/R.

What's more, given that PPI network was too large to be analyzed, we conducted clustering analysis and screened out four modules, followed by the protein domains enrichment. The protein domains in Module 4 significantly enriched in Transient receptor potential channel, and canonicaltransient receptor potential channel 6 (TRPC6) was among the genes in this module. TRPC6 have been reported to be associated with I/R in the lung [34], brain I/R injury is reported to be prevented through TRPC6-MEK-CREB and TRPC6-CaMKIV-CREB pathways [35], and activating TRPC channels before ischemia is believed to have early neuroprotective effects on retinal ganglion cells in vitro [36]. In this study, log FC for TRPC6 between normal and I/R injury rats samples was 1.53, indicating that TRPC6 was an up-regulated DEG. Currently, there have been no reports showing the relations between I/R injury in kidney and TRPC6, therefore, our finding may provide new evidence.

Only 6 samples were available for each rat strain in this bioinformatics analysis. The sample size is relative small, thus, further analysis based on a larger sample size should be performed.

Conclusion

The potential mechanism of I/R injury in kidney in rats model was investigated in our present research. Our findings suggested that several GO terms, pathways and protein domains may be interrupted during the disease progress. Meanwhile, miR-29c and TRPC6 were suggested to be potential novel targets for this disease. Our study revealed the underlying mechanism of I/R from several levels and obtained some new results. These findings have the potential to investigate the pathogenesis of I/R injury.

Conflict of Interests

All authors declare that they have no conflict of interest.

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