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*Original Article*

# Biological mechanism analysis of acute renal allograft rejection: integrated of mRNA and microRNA expression profiles

Shi-Ming Huang[1], Xia Zhao[2], Xue-Mei Zhao[3], Xiao-Ying Wang[4], Shan-Shan Li[5], Yu-Hui Zhu[5]

*Departments of [1]Urology, [2]Nephrology, [3]Anorecta, Qianfoshan Hospital Affiliated to Shandong Univercity, Jinan 250014, China; [4]Chemical Defense Clusters Medical Teams of 74122 PLA Troops, Jinan 250031, China; [5]Department of Nephrology, The 456th Hospital of Jinan Military Region, Jinan 250031, China*

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**Abstract:** Objectives: Renal transplantation is the preferred method for most patients with end-stage renal disease, however, acute renal allograft rejection is still a major risk factor for recipients leading to renal injury. To improve the early diagnosis and treatment of acute rejection, study on the molecular mechanism of it is urgent. Methods: MicroRNA (miRNA) expression profile and mRNA expression profile of acute renal allograft rejection and well-functioning allograft downloaded from ArrayExpress database were applied to identify differentially expressed (DE) miRNAs and DE mRNAs. DE miRNAs targets were predicted by combining five algorithm. By overlapping the DE mRNAs and DE miRNAs targets, common genes were obtained. Differentially co-expressed genes (DCGs) were identified by differential co-expression profile (DCp) and differential co-expression enrichment (DCe) methods in Differentially Co-expressed Genes and Links (DCGL) package. Then, co-expression network of DCGs and the cluster analysis were performed. Functional enrichment analysis for DCGs was undergone. Results: A total of 1270 miRNA targets were predicted and 698 DE mRNAs were obtained. While overlapping miRNA targets and DE mRNAs, 59 common genes were gained. We obtained 103 DCGs and 5 transcription factors (TFs) based on regulatory impact factors (RIF), then built the regulation network of miRNA targets and DE mRNAs. By clustering the co-expression network, 5 modules were obtained. Thereinto, module 1 had the highest degree and module 2 showed the most number of DCGs and common genes. TF CEBPB and several common genes, such as RXRA, BASP1 and AKAP10, were mapped on the co-expression network. C1R showed the highest degree in the network. These genes might be associated with human acute renal allograft rejection. Conclusions: We conducted biological analysis on integration of DE mRNA and DE miRNA in acute renal allograft rejection, displayed gene expression patterns and screened out genes and TFs that may be related to acute renal allograft rejection.

**Keywords:** Renal transplantation, acute rejection, microRNA, mRNA, transcription factor

## Introduction

Renal transplantation is the best choice for most patients with end-stage renal disease [1], such as renal cancer and kidney injury, which has extended and improved the living quality for the majority of patients [2]. Although the therapeutic methods achieving long-term graft survival have improved, the immune response is still a common trouble in renal graft recipients. Acute rejection is a major immunologic risk factor for graft failure [3, 4], which might lead to severe renal injury. It was reported that renal allograft failure was the fourth most common cause of end-stage renal disease in United States [5]. In order to impede the immune response, most graft recipients need lifelong treatment with potent immunosuppressive drugs which have many side effects. Currently, the diagnosis of acute rejection mainly uses histological features of the allograft biopsy [4]. Limited knowledge of the molecular pathogenesis is a major obstacle in the identification of drug targets and development of therapeutic strategies for acute renal allograft rejection.

MicroRNAs (miRNAs) are a class of small (only 18-25 nucleotides), endogenous, non-coding

RNAs that regulate posttranscriptional gene expression by translational repression or mRNA degradation [6]. In fact, approximately one-third of human mRNAs may be regulated by miRNAs [7, 8]. miRNAs are involved in gene regulation in different processes such as physiological [9] and pathophysiological processes [10]. It was reported that an important role for miRNAs was regulating the development of immune cell and modulating the immune responses [11-14]. Recently, differential expression of miRNAs in several diseases suggested that they might have key regulatory roles in a wide range of biological processes [15, 16]. Thus, miRNAs have been considered as potential therapeutic biomarkers [17] as well as mRNAs.

With recent advances in immunology and transplantation biology, the development of reliable assays is urgently needed to allow us to identify and predict the development of immunologic graft rejection. High throughput microarray technology, promised as a clinical tool, provides methods to study disease-specific transcriptional changes simultaneously. Human miRNA and mRNA expression profiling associated with transplant rejection and injury have been already reported [2, 18, 19]. Previous researches have illuminated variations in gene expression in allograft biopsy samples from patients with acute rejection [19-21], and demonstrated a few immunologic relevant gene associated with acute rejection and clinical outcomes. Those existing data which deposited in public-available repositories, such as ArrayExpress Archive, recently can provide the secondary use to predict outcome and biomarkers of acute rejection in renal allografts for us.

It is on the cards that a single miRNA influences expression of multiple different target genes or different miRNAs control a single mRNA target [22-24]. In presently, the authentication of miRNA target genes have gained extensive attentions. Many bioinformatic prediction strategies developed rapidly based on the confirmed rules of interaction between miRNAs and their targets. However, to date, miRNA targets are unstable using different programs, and so rare confirmed [25].

Although acute rejection in renal allograft have been profiled extensively by genomics-based studies [2, 3, 19], little is known about the influence of miRNA-target interactions and how the global expression alterations form a network. Moreover, almost all previous studies researched the differential expression between acute rejected renal allograft and normal biopsies, however, few studies compared the differential expression between acute rejected allograft and well-functioning renal allograft.

To better understand the complex pathology associated with acute rejection in renal allograft and improve the early diagnosis of acute rejection, study on molecular mechanism of it is urgent. In this study, we extracted a miRNA expression profile and a mRNA expression profile respectively, and identified the differentially expressed (DE) miRNAs and the DE mRNAs. In order to improve the accuracy of miRNA targets prediction, five algorithms were combined simultaneously. Then, compared with the miRNA targets and DE mRNAs, overlapped genes were selected as common genes, which were considered as more robust genetic markers and were more beneficial to the diagnosis and treatment for acute renal allograft rejection. Next, we took a systematic approach to investigate DE genes between acute rejection allograft and well-function allograft, included co-expression network, cluster analysis and functional enrichment analysis. In a word, we may provide information for understanding the underlying molecular mechanisms of acute rejection, and at the same time provide methods for advanced diagnostics and prognosis.

## Material and methods

### *Identification of DE miRNAs and DE mRNAs*

The microarray miRNA and mRNA expression profiles of renal allograft were downloaded from ArrayExpress (http://www.ebi.ac.uk/arrayexpress/) database under access number of E-GEOD-30282 [18] and E-GEOD-1563 [26]. In E-GEOD-30282, the miRNA expression profiles of 51 samples (including 41 acute renal allograft rejection cases and 10 well-functioning transplants as controls) were selected, and samples with delayed graft function were excluded. In E-GEOD-1563, 17 samples (including 7 acute renal allograft rejection cases and 10 well-functioning transplants as controls) were selected, and samples included donor, renal dysfunction without rejection and peripheral blood originated were excluded.

We provided the Oligo package supported by Affymetrix to preprocess the expression chips. The two comparison experiments were conducted by Linear Models for Microarray Data (LIMMA) package. DE miRNAs and mRNAs were identified by assimilating a set of gene-specific *t* tests with the threshold of false discovery rate (FDR) ≤ 0.05 and logFC > 2.

*miRNA target genes prediction*

The DE miRNAs identified in the previously step were used for predicting target genes. Since miRNA targets were unstable using different algorithms, five algorithms including miRanda [27], miRDB [28], miRWalk [29], RNA22 [30] and Targetscan [31] were employed to improve the reliability of miRNA targets prediction in this study. A gene was identified as target gene only if the gene was confirmed by at least four algorithms (SUM ≥ 4). Target genes would be selected as common genes if they overlapped with the DE mRNAs in E-GEOD-1563. Below, these methods were explained in detail.

*miRanda:* Originally, miRanda was used to find miRNA targets in *Drosophila* by Enright AJ et al. [27], and was developed to predict targets in humans subsequently. As one earlier miRNA target predictor, miRanda uses a three-phase method for target analysis [27]. First, the miRNA sequences are scanned against 3' untranslated region (UTR) to check for whether two sequences are complementary using a position-weighted local alignment algorithm. Second, the free energy of each miRNA: UTR is calculated. Finally, evolutionary conservation is used as a final informational filter. The targets are scored based on how well they match the miRNAs. A predicted target is ranked high in the results by obtaining a high individual score or having multiple predicted sites.

*miRDB:* miRDB was developed by Wang X et al., which was an online database system for miRNA target prediction and functional annotation (http://mirdb.org) [28]. For convenience in application, genome-wide target prediction was performed, and the predicted targets were preloaded into miRDB. 1437 miRNAs targeting 47946 unique genes are contained in miRDB version 2.0 in five species (human, mouse, rat, dog, and chicken). Flexible web query interface is developed to retrieve target prediction results, which is sorted by target score. The detailed results contain information about the miRNAs, the targets and their 3'-UTR sequences.

*miRWalk:* miRWalk was first presented by Dweep H et al. [29]. It is a comprehensive database (http://mirwalk.uni-hd.de/) that can be used to predict all the possible miRNA binding sites by "walking" the genes of three genomes (human, mouse and rat). This algorithm is based on a computational approach to identify multiple consecutive complementary subsequences between miRNA and all download sequences. Then the results are performed comparison with the results obtained from other established prediction programs, and validation by performing an automated text-mining search in the titles/abstracts of the PubMed articles. The predicted and validated information is stored in miRWalk database.

*RNA22:* RNA22, presented by Miranda KC et al., was a pattern-based method for identifying miRNA binding sites and their corresponding miRNA/mRNA complexes [30]. It first finds putative miRNA binding sites in the sequence of interest without a need to know the identity of the targeting miRNA. RNA22 identifies target islands and evaluates the free energy of paired target islands and candidate miRNAs, and experimentally evaluates selected miRNA/target-island interactions.

*Targetscan:* To identify the targets of mammalian miRNAs, Lewis BP et al. developed the Targetscan algorithm, which combined thermodynamics-based modeling of RNA: RNA duplex interactions with comparative sequence analysis to predict miRNA targets conserved across multiple genomes [31]. The software is available for download at http://genes.mit.edu.libproxy.tulane.edu:2048/targetscan. The specific methods are detailed in previous study [31].

*Identification of co-expression network and modules*

Based on the viewpoint of systems biology, genes with similar functions are frequently co-expressed across a set of samples [32-34]. In this process, we applied the Differentially Co-expressed Genes and Links (DCGL) 2.0 package in R to identify differentially co-expressed genes and links from mRNA microarray data [35]. Differential co-expression profile (DCp) and differential co-expression enrichment (D-

**Table 1.** The number of predicted target genes by different target gene prediction algorithms

| miRNA | miRanda | miRDB | miRWalk | RNA22 | Targetscan | SUM ≥ 4 |
|---|---|---|---|---|---|---|
| hsa-miR-150 | 348 | 222 | 346 | 151 | 349 | 349 |
| hsa-miR-155 | 358 | 305 | 360 | 82 | 361 | 361 |
| hsa-miR-139-5p | 197 | 197 | 197 | 0 | 197 | 197 |
| hsa-miR-124 | 354 | 354 | 354 | 0 | 354 | 354 |
| hsa-miR-1180 | 9 | 9 | 9 | 0 | 9 | 9 |
| has-miR-424* | 0 | 0 | 0 | 0 | 0 | 0 |

Ce), involved in differential co-expression analysis (DCEA) module which was one of the four modules in DCGL 2.0 package, were used to identify differentially co-expressed genes (DCGs) and differentially co-expressed links (DCLs) [36]. In our study, we implemented DCsum, i.e. the intersection of DCp- and DCe-derived DCGs, to filtrate the DCGs. To improve the dependability of DCGs, we filtered the top 25% of genes with the absolute value of correlation coefficient higher than 0.7.

In the network, regulatory impact factors (RIF) metric was applied to identify the key transcription factors (TFs). RIF metric was originally developed to identify causal regulators from gene expression data contrasting 2 conditions (e.g. healthy vs. disease) [37]. RIF can combine the change in co-expression between TFs and DE genes. Then the co-expression network of the DE mRNAs with RIF value was constructed.

To explore the highly interconnected regions in the network, we implemented the cluster analysis to the network using ClusterONE plugin of Cytoscape software. Nodes represented biomolecules and edges indicated functional interactions between molecules in a molecular network.

*Functional enrichment analysis*

Gene Ontology (GO) database and Kyoto Encyclopedia of Genes and Genomes (KEGG) database provide systematic analysis of gene functions by computerizing current knowledge on cellular processes and standardizing gene annotations [38, 39]. In this study, to investigate the functions and enriched pathways of these DCGs, GO functional enrichment and KEGG pathway enrichment analysis for DCGs were performed using the online tool DAVID [40] (http://david.abcc.ncifcrf.gov/tools.jsp). In DAVID, the significant categories were identified by EASE score. The threshold of EASE score < 0.05 and the minimum number of genes for the corresponding term > 2 were considered significant for a category.

## Results

*Identification of DE miRNAs, DE mRNAs, miRNA targets and common genes*

In E-GEOD-30282, we got 6 DE miRNAs, including two up-regulated miRNAs (miRNA-150 and miRNA-155) and four down-regulated miRNAs (miRNA-1180, miRNA-124, miRNA-169-5P and miRNA-424*). Based on these DE miRNAs, 1270 target genes (included 710 up-regulated and 560 down-regulated target genes) were predicted using five target gene prediction algorithms with SUM ≥ 4 (**Table 1**).

In E-GEOD-1563, we got 698 DE mRNAs under the criterion of FDR ≤ 0.05, including 138 up-regulated genes and 560 down-regulated genes. Overlapping the miRNA target genes and DE mRNAs, 59 genes were identified as common genes. The common genes were selected for further research.

*Identification of co-expression network and modules*

We applied the DCGL 2.0 package in R to identify DCGs and DCLs in acute renal allograft rejection cases and well-functioning allograft from mRNA microarray data. A total of 103 DCGs were identified by DCp and DCe methods with the absolute value of correlation coefficient higher than 0.7. In RIF metric, we obtained 5 genes with RIF value which were TFs. The 5 TFs were ARID5B, MECOM, NFE2L1, STAT1 and CEBPB, thereinto ARID5B had no corresponding target and only CEBPB mapped on the co-expression network. The co-expression network of the DCGs was shown in **Figure 1**.

Biological network is considered as a scale-free network whose degree distribution follows a power law in the real network. The scale-free property strongly correlates with the network's robustness. According further analysis, we found that our co-expression network was confirmed to the scale-free network whose degree

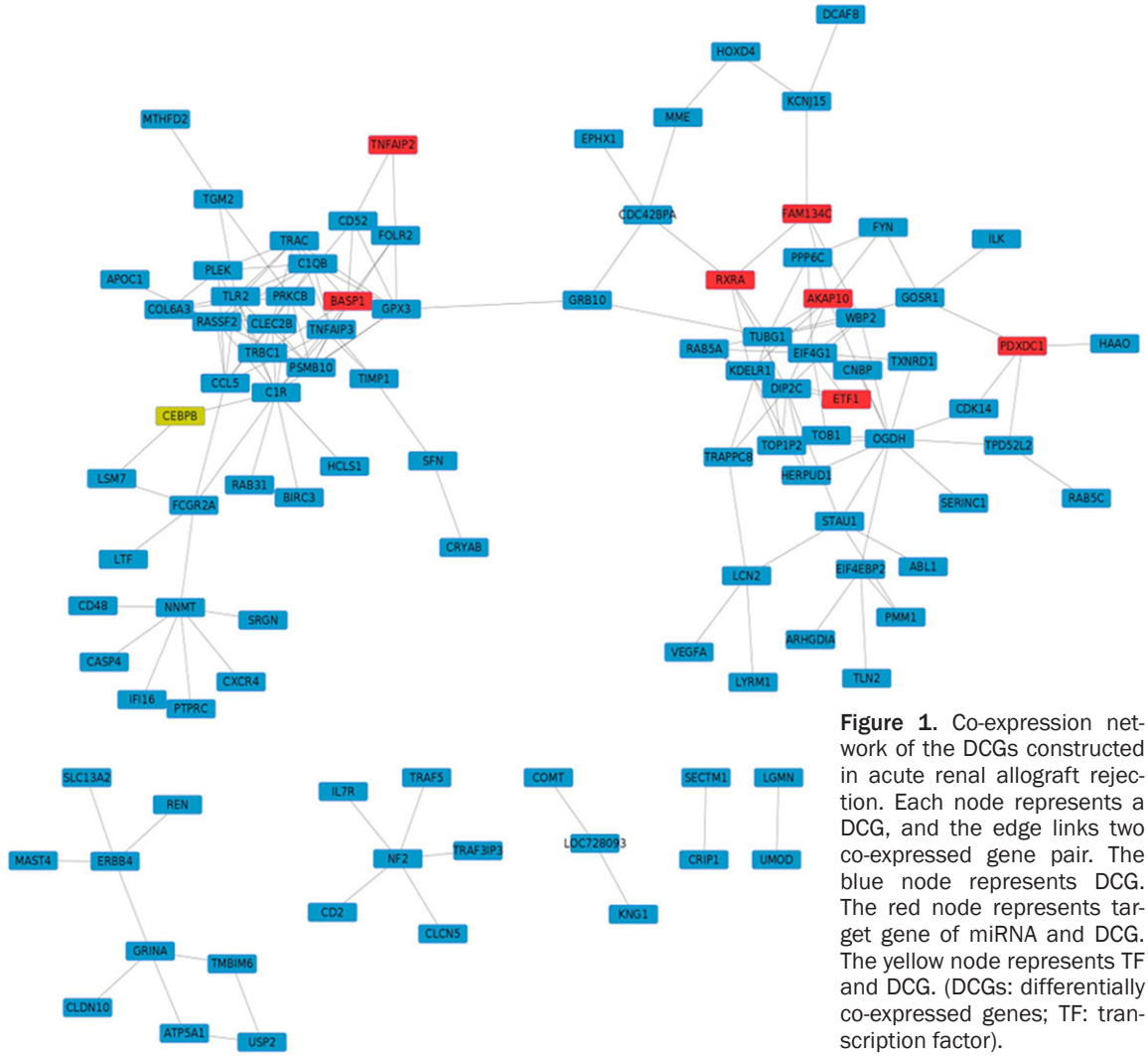


**Figure 1.** Co-expression network of the DCGs constructed in acute renal allograft rejection. Each node represents a DCG, and the edge links two co-expressed gene pair. The blue node represents DCG. The red node represents target gene of miRNA and DCG. The yellow node represents TF and DCG. (DCGs: differentially co-expressed genes; TF: transcription factor).

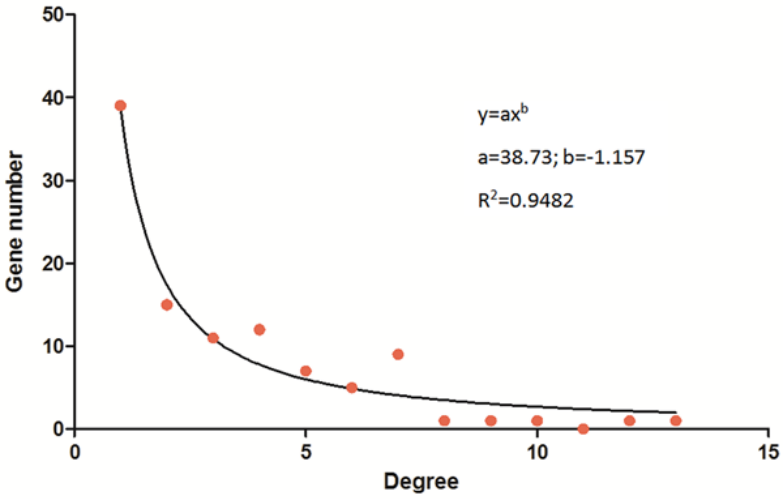


**Figure 2.** The scattergram of gene degree in co-expression network. The co-expression network was a scale-free network whose degree distribution followed a power law ($y = ax^b$, where a = 38.73, b = -1.157).

distribution followed a power law ($y = ax^b$, where a = 38.73, b = -1.157; $R^2$ = 0.9482) (**Figure 2**).

In a scale-free network, the vertice with a degree greatly exceeds the average. The nodes with high degree are often called 'hubs' or 'old' nodes which are thought to serve crucial importance. There were several 'hubs' in this co-expression network, such as C1R (degree = 13), OGDH (degree = 12) and TUBG1 (degree = 10). Common genes, such as BASP1, AKAP10 and RXRA, also showed relative high degree.

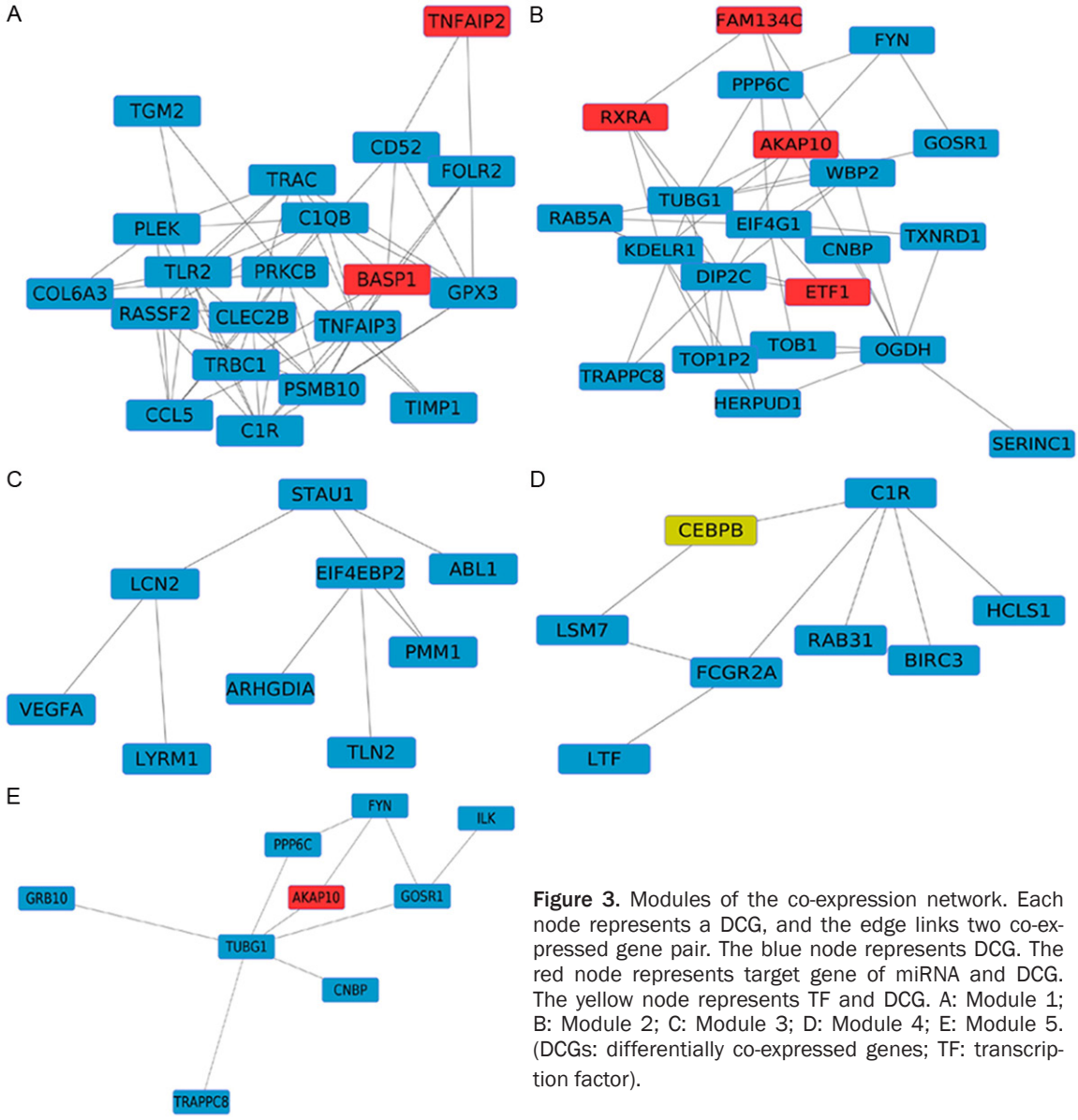


**Figure 3.** Modules of the co-expression network. Each node represents a DCG, and the edge links two co-expressed gene pair. The blue node represents DCG. The red node represents target gene of miRNA and DCG. The yellow node represents TF and DCG. A: Module 1; B: Module 2; C: Module 3; D: Module 4; E: Module 5. (DCGs: differentially co-expressed genes; TF: transcription factor).

ClusterONE plugin of Cytoscape software was used to extract modules. We got 5 modules that were shown in **Figure 3**. Module 1 had the highest degree and it included 2 target genes of DE miRNAs (**Figure 3A**). Module 2 had the most number of gene and included 4 target genes (**Figure 3B**). The TF CEBPB was mapped on Module 4 (**Figure 3D**). Module 3 and 5 contained fewer DCGs with lower degree (**Figure 3C** and **3E**).

*Pathway enrichment analysis*

To annotate the 103 DCGs, pathway analysis based on KEGG was preformed. These DCGs were significantly yielded in focal adhesion ($P$ = 0.011) and prion diseases ($P$ = 0.043). By GO analysis, it was found that these genes mainly participated in bioprocess of regulation in apoptosis ($P$ = 6.19E-7), programmed cell death ($P$ = 7.19E-7), and cell death ($P$ = 7.60E-7).

## Discussion

Renal transplantation, which was defined as the organ transplant of a kidney into a patient with end-stage renal disease, was firstly preformed on 1950 in the United States. Unfortunately, the donated kidney was rejected because of lack of immunosuppressive drugs. Although renal transplantation has progressed from a risky experimental therapy to a safe and

life-saving treatment, acute immune rejection of the allograft is still a common issue for allograft recipients. At presently, the specific pathogenesis is still unclear. A deeper understanding of molecule mechanisms of acute rejection may help us to identify diagnostic methods and new targets for therapy.

In this study, we got 698 DE mRNAs between renal allograft with acute rejection and well-functioning allograft. Meanwhile, 2 up-regulated and 4 down-regulated miRNAs were revealed. Previous studies of acute renal allograft rejection demonstrated that there were significant differences in the expression of multiple genes [21, 41-43]. Li B. et al. demonstrated that the expression of mRNAs encoding cytotoxic attack proteins granzyme B and perforin was increased in human acute renal allograft rejection [42]. Muthukumar T. et al. reported a noninvasive means of measurement of FOXP3 mRNA in urine to improve the prediction of outcome of acute renal allograft rejection [41]. Over the past years, the role of miRNAs in predicting allograft rejection has been investigated by several research groups and still is a topic of interest. However, the published results showed a significant heterogeneity. Anglicheau et al. used miRNAs expression profiles to predict human renal allograft status, and investigated that miR-142-5p, -155, -223 were overexpressed in acute rejection biopsies [44]. In the Wilflingseder paper, 10 up-regulated miRNAs and 18 down-regulated ones were found [18]. Sui W et al. demonstrated that 20 miRNA were differentially expressed in acute rejection samples and normal controls [19].

Remarkably, different from previous studies in which normal biopsies were selected as control group, our study considered well-functioning renal allograft as controls when detecting DE genes. When comparing acute rejected renal allograft with well-functioning allograft, it will be more conducive to understand the molecule pathogenesis of acute rejection, develop accurate therapeutic strategies for acute renal allograft rejection, and at the same time improve success rate of renal transplantation.

In order to gain more robust genetic markers of human renal allograft rejection, we improved our study in two ways: the improvement of miRNA targets predictive accuracy by combining multiple bioinformatic strategies and the acquirement of common genes between miRNA targets and DE mRNAs. In this study, we predicted the target genes of DE miRNAs associated with human renal allograft rejection and well-functioning allograft. Comparing with DE mRNAs, we obtained 59 common genes, and seven of them were mapped on the co-expression network.

RXRA (degree = 5), one of the common genes in module 2, was included in pathway in cancer. Previous studies had shown the biological significance of RXRA in a variety of diseases, such as Alzheimer's disease [45], colon adenoma [46], tetralogy of Fallot [47] and dyslipidemia [48, 49]. Studies of vitamin D pathway showed that RXRA polymorphisms were associated with increased renal cell carcinoma risk [50, 51]. However, little is known whether RXRA plays a role in human acute renal allograft rejection. Our study found that RXRA was included in the terms of defense response, response to wounding and acute inflammatory response by the functional analysis. Combined with the significant role in co-expressed network, we predicted that RXRA might be related to acute renal allograft rejection.

In the co-expression network, CEBPB, as the only TF mapped on the network, was directly connected to C1R and LSM7. A large amount of target genes of CEBPB had been confirmed by many scientists, such as IL-6 [52], TNF-alpha [53], ABCC2 [54] and CREB1 [55]. Yu et al. predicted 106 potential CEBPB target genes in acute promyelocytic leukemia induced by all-trans retinoic acid using high-throughput approach [56]. A new study in mice found that in the case of CEBPB deficiency, musculoskeletal phenotypes would show the symptom of cleidocranial dysplasia [57]. Tsutsui T et al. indicated that CEBPB could regulate many target genes associated with immune response, such as PRMT5 and DNMT3A [58]. C1R as the CEBPB target gene showed the highest degree in the co-expression network (degree = 13), indicating that C1R played an important role for the co-expression network. C1R gene encodes a protein named C1r protease which belongs to complement component [59]. A study of C1R found that its deficiency was associated with cutaneous and renal disease because of the impairment of bactericidal activity and immune adherence [60]. It was also reported that C1R

was significantly expressed in both autografted and allografted skins in a few days after skin graft in mouse, and showed that C1R might be associated with the graft versus host immune responses in mouse [61]. So CEBPB and C1R were also predicted as genes associated with acute renal allograft rejection.

In present study, clustering was used to build groups of co-expressed genes, in which contained functionally related genes, such as genes that were co-regulated, or genes in a specific pathway. In our research, we got 5 modules. Thereinto, module 1 showed the highest degree with 2 common genes. Module 2 had the most number of DCGs and common genes. Maybe module 1 and module 2 played more important role in acute renal allograft rejection.

## Conclusion

In our study, we identified DE mRNAs and DE miRNAs between acute rejected renal allograft and well-functioning allograft, respectively. Then, the DE miRNA target genes were predicted by combining five algorithms. Next, we obtained 59 common genes by overlapping DE mRNAs and target genes of DE miRNAs between renal allograft with acute rejection and well-functioning allograft. Co-expression network of DCGs was constructed, and TF CEBPB and several common genes were mapped on the co-expression network. They were predicted to be associated with human acute renal allograft rejection. One limitation of this study is that DCGL 2.0 package used to co-expression analysis is only suitable for individual microarray dataset, thus, this limits the integration of multiple studies in present study. In conclusion, this study can help us to understand the pathogenesis of human acute renal allograft rejection which was helpful for the diagnosis and treatment of human acute renal allograft rejection.

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## Disclosure of conflict of interest

None.

**Address correspondence to:** Dr. Yu-Hui Zhu, Department of Nephrology, The 456th Hospital of Jinan Military Region, NO. 25 Wuyingshan Road, Jinan 250031, China. Tel: 86-0531-58863638; E-mail: yuhuizhu123@yeah.net

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