Noninvasive Identification of Core Gene Biomarkers in Patients with Acute Kidney Transplant Rejection

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Abstract

Background: Acute rejection (AR) is one of common and critical complications after kidney transplantation, which gives rise to an increasing of allograft loss and even death. However, the potential mechanisms of AR remain incompletely understood at present. This study aimed to reveal the underlying mechanisms and identify core biomarkers of AR.

Methods: The AR gene expression profile GSE1563 involving 6 renal transplant patients undergoing AR and 9 patients with well-functioning transplant with no clinical evidence of rejection was selected to analyze the differentially expressed genes (DEGs) from purified RNA of peripheral blood lymphocytes. DAVID was used to carry out Gene ontology (GO) and the Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway analyses. A protein-protein interaction (PPI) network was built to display the interactions among these DEGs. To get further reliable significant genes and mechanisms, gene set enrichment analysis (GSEA) was applied to evaluate the hub gene analyzing via PPI network.

Results: A total of 347 DEGs were captured, including 301 upregulated genes and 46 downregulated genes. Go and KEGG pathway analysis showed the DEGs were particularly enriched in immune response, inflammatory response to antigenic stimulus, RNA transport and protein stabilization. The PPI network indicated 3 modules were also mainly involved in immune response and RNA transport. 18 hub genes were selected in PPI network, among which there were 6 core genes evaluated by DAVID. By the way of GSEA, Oxidative stress was another potential mechanism besides immunity and ncRNA transport.

Conclusion: Our analysis uncovered the immune response including humoral and cellular immunity, ncRNA transport as well as oxidative stress was the major mechanisms of AR associated respectively with MAPK8, CCL5, HMGB1, NCBP2, XPO1 and APP, which could be novel noninvasive biomarkers in peripheral blood for early diagnosis and could be helpful to the treatment of AR.

1. Introduction

Kidney transplantation is a crucial therapy for patients with end-stage kidney disease (ESKD), as it enhances the quality of patients' life and reduces mortality compared with dialysis^[1]. Due to the usage of advanced immunosuppressive medications and the improvement of organ procurement methods, the 5-year deceased donor kidney allograft survival rates have increased up to 72.4%^[2]. However, the risk of allograft loss, especially acute graft rejection remain a major hurdle to overcome^[3]. In a study of a consecutive series of 319 cases receiving deceased kidney transplantations over a 7 year period, 31% experience a single early acute rejection^[4]. The potential mechanisms of acute rejection (AR) following kidney transplantation remain unclear. In this context, it is elementary to promote our understanding between immune system and the transplanted organ to immune and non-immune mechanisms of injury.

Generally, the gold standard for diagnosis of AR is histology obtained via needle biopsy, whereas this technique is infrequently used for surveillance as a result of cost, potential complications, patient

discomfort and inconvenience^[5]. Furthermore, other laboratory examinations involving serum creatinine, proteinuria and donor-specific antibodies as well as some non-invasive tests like renal ultrasound with doppler for renal arterial and venous indices also can help to evaluate the condition of AR. However, these methods for AR diagnosis display poor specificity and efficiency, which are difficult for us to gain accurate diagnosis and proper treatment early, so that some patients with AR lost the best chance for therapy, thereby causing graft dysfunction and even improving the death risk^[6]. Hence, the identification of specific and sensitive biomarkers is essential to assist us accurate diagnosis and treatment of AR as soon as possible, especially noninvasive biomarkers.

High-throughput gene microarray and bioinformatic analysis have enabled us to screen molecular markers among healthy individuals and patients, which provides novel perceptions into diseases at multiple levels ranging from the variation of copy number at the genome level to gene expression at transcriptome and protein level, and even epigenetic alterations^[7]. However, the application of microarrays in clinic is limited due to overwhelming number of genes identified by gene profiling, shortage of repeatable and independent validation and complex statistical analyses^[8]. Moreover, the majority of the microarrays are grounded on the genes in tissues which is hard to verify and test except by invasive methods^[9]. As a result, for the sake of put these expression profiles into clinical applications as quickly as possible, it is vital to confirm a suitable amount of serum genes and develop an appropriate and perfect way which can be worked out by routine assay.

In this study, the gene expression profile of GSE1563 was downloaded from the Gene Expression Omnibus (GEO, <http://www.ncbi.nlm.nih.gov/geo/>) which is an online public collection database for microarray data and used GEO2R online software to thoroughly identify differentially expressed genes (DEGs) between kidney transplantation patients with and without AR. Then, we analyzed gene ontology (GO) including biological processes (BP), molecular function (MF), cellular component (CC), and KEGG pathway of the DEGs. Furthermore, we constructed protein-protein interaction (PPI) network of these DEGs and picked up the top 12 hub genes with a high degree of connectivity. Subsequently, we carried out three modules and confirmed their enriched pathway. After the analysis of hub genes, we sustained the enrichment of hub genes to find the pivotal mechanism of AR according to Gene Set Enrichment Analysis (GSEA).

2. Materials And Methods

2.1 Microarray Data we downloaded the gene expression profile of GSE1563 from the National Center for Biotechnology Information (NCBI) GEO database, a free and publicly available database^[10]. We selected a total of 15 samples of purified RNA of peripheral blood lymphocytes (PBL), involving 6 renal transplant patients undergoing AR and 9 patients with well-functioning transplant with no clinical evidence of rejection, which was based on GPL8300 platform [HG_U95Av2] Affymetrix Human Genome U95 Version 2 Array by Flechner SM et al. We also obtained the Series Matrix File of GSE1563 from the GEO database in pubmed. Inclusion criteria of AR group for this microarray was according to Banff criteria and confirmed

by response to anti-rejection therapy^[11]. Transplant patients with CMV or other infections in clinical and laboratory evidence were excluded. Immunosuppression medications contained a calcineurin inhibitor or sirolimus, with mycophenolate mofetil and steroids. Additionally, patients with no rejection were in line with more than one-year post transplant in patients with good transplant function and normal histology. Genes expression profile was evaluated by Affymetrix HG-U95Av2 GeneChip arrays via total RNA extracted from all samples.

2.2 Screen Genes of Differential Expression The screening of DEGs between AR and no rejection samples was performed by using GEO2R(<http://www.ncbi.nlm.nih.gov/geo/geo2r>), which is a online analysis tool for the GEO database according to R language. Compliance with the previous criteria^[12–13], we regulated DEGs as differentially expressed with $\log FC > 1$ (upregulated genes) or $\log FC < -1$ (downregulated genes). In the meantime, the adjusted P value < 0.05 was regarded statistically significant to reduce the false positive rate. Moreover, after downloading the correlative data of TXT files, we employed visual hierarchical cluster analysis to show the heatmap and volcano plot of AR and no rejection samples via ImageGP (<http://www.ehbio.com/ImageGP/index.php/Home/Imdex/index.html>)

2.3 Gene ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) Pathway Analysis of DEGs GO analysis is a public framework which can note on genes and gene products involving biological pathways (BP), molecular function (MF) and cellular components (CC)^[14]. The KEGG pathway is a set of databases manifesting genomes and biological pathways related to diseases and drugs. KEGG is essentially a resource to get an systematic understanding of biological functions and even certain high-level genome information^[15]. In our study, the GO and KEGG analysis was accomplished by the database for Annotation, Visualization and Integrated Discovery (DAVID, <http://david.ncifcrf.gov>) (version 6.7), which has contained a great many biological data and concerned analysis methods, and then supplied tools about the biological function annotation information for lots of genes or proteins^[16]. $P < 0.05$ was regarded as the cutoff criterion with significant difference. Besides, we displayed the scatter plot of the BP, MF, CC and KEGG pathway of these DEGs using ImageGP.

2.4 Protein-Protein Interaction(PPI) Network and Module Analysis We used the PPI network to illustrate the core hub genes and main gene modules between transplant patients with and without AR. Search Tool for the Retrieval of Interacting Genes (STRING) is an well-known database to assess and integrate the PPI information(<http://www.stringdb.org/>), which includes physical and functional associations. It covered 24584628 proteins from 5090 organisms in STRING version 11.0^[17]. We applied DEGS to identify the interactional associations among them using STRING. Furthermore, we built a PPI network through Cytoscape software platform. Then, we regarded the maximum number of interactors = 0, confidence score ≥ 0.4 as the curoff criterion. Moreover, the Molecular Complex Detection (MCODE) app was perfomed to reveal modules of the PPI network in Cytoscape in accordance with degree cutoff = 2, k-core = 2, node score cutoff = 0.2, and max. depth = 100. Top 12 hub genes were selected in line with the ranking order of connectivity degree via Cytoscape with maximum number of interactors ≤ 5 and

confidence score ≥ 0.4 . Besides, GO and KEGG pathway analyses were applied to demonstrate the potential information.

2.5 Gene Set Enrichment Analysis (GSEA) 6 AR samples from GSE1563 were divided into two groups (high vs. low) on the basis of expression level of several hub genes, and median expression value was considered as the cutoff point. In order to reveal the potential function of these related hub genes, GSEA(<http://software.broadinstitute.org/gsea/index.jsp>) was performed between the two groups. Gene sets database c2.cp.kegg.v7.1.symbols.gmt, c5.bp.v7.1. symbols.gmt, c5.cc.v7.1. symbols.gmt, c5.mf.v7.1. symbols.gmt were selected as the reference gene sets. $FDR < 0.05, |\text{enrichment score}(ES)| > 0.5$ and gene size ≥ 100 were regarded as the cutoff criteria.

2.6 Statistical Analysis All obtained data were reported as means $\pm$ SD(standard deviation) Statistical significance was assessed by the two tailed Student's t-test via SPSS 19.0 software. A difference of $P < 0.05$ was considered statistically significant.

3. Results

3.1 Identification of DEGs In our study, a total of 6 kidney transplant patients undergoing AR and 9 patients with well-functioning transplant with no clinical evidence of rejection were analyzed. We used the GEO2R online analysis tool on the basis of default parameters to screen the DEGs with $\log FC \leq -1$ or $\log FC \geq 1$ and adjusted P value < 0.05 and as the cut-off criteria. Through analysis of GSE1563, a total of 347 DEGs were captured, 301 of which were upregulated genes while 46 were downregulated genes. The expression ratio of these DEGs were shown in the volcano plot (Fig. 2A). Meanwhile, the top30 upregulated genes and top30 downregulated genes between transplant patient with and without AR were displayed in the heatmap (Fig. 2B). In these 347 DEGs, the top5 up-regulated genes including CD9, SLC39A6, ATP5F1, MORF4L2 and RYK as the top5 down-regulated genes were MAPK8, HLCS, CCL7, CCL5 and S100A9. The gene titles and biological functions of these 10 genes were displayed in Table 1.

DEGs	Gene title	Gene symbol	LogFC	Biological function
Up-regulated	CD9 molecule	CD9	1.90	Cell differentiation, adhesion, and signal transduction
	solute carrier family 39 member 6	SLC39A6	1.88	Zinc transport and lymphocyte activation
	ATP synthase, H ⁺ transporting, mitochondrial F0 complex, subunit B1	ATP5F1	1.86	Participate in ATP synthesis
	mortality factor 4 like 2	MORF4L2	1.84	abnormal nuclear morphology and cell death.
	receptor like tyrosine kinase	RYK	1.77	stimulating Wnt signaling pathways
Down-regulated	S100 calcium binding protein A9	S100A9	-1.51	Regulating cell cycle progression and differentiation
	C-C motif chemokine ligand 5	CCL5	-1.50	release of histamine from basophils and activating eosinophils
	C-C motif chemokine ligand 7	CCL7	-1.48	Involving in inflammation and metastasis
	holocarboxylase synthetase	HLCS	-1.42	gluconeogenesis, fatty acid synthesis and branched chain amino acid catabolism
	mitogen-activated protein kinase 8	MAPK8	-1.33	T cell proliferation, apoptosis and differentiation

Table 1

Top5 up-regulated and down-regulated differentially expressed gene in patients with AR

3.2 GO Enrichment Analysis To obtain a more particular and in-depth knowledge of these captured DEGs, we applied DAVID to analyze significantly enriched GO function of these upregulated DEGs and downregulated DEGs. The results displayed that the up-regulated DEGs in BP were mainly enriched in protein ubiquitination, protein stabilization, response to drug, chemokine-mediated signaling pathway and inflammatory response to antigenic stimulus whereas the downregulated DEGs in BP were enriched in innate immune response, immune response, neutrophil chemotaxis, cellular response to interleukin-1 and positive regulation of inflammatory response. Concerning MF, the up-regulated DEGs were primarily enriched in poly(A) RNA binding, ATP binding, nucleotide binding, protein kinase activity and transcription corepressor activity while the down-regulated DEGs were enriched in protein binding, Toll-like receptor 4 binding, arachidonic acid binding, C-C motif chemokine receptor 1 (CCR1) chemokine receptor binding and the receptor of advanced glycation end-products (RAGE) receptor binding. Besides, CC analysis

indicated that the up-regulated DEGs were principally enriched in cytoplasm, nucleus, extracellular exosome, nucleoplasm and nucleolus. The down-regulated DEGs were related to extracellular space, cytosol, extracellular region, extracellular exosome and cytoskeleton (Table 2&Figure 3A, B **and C**).

Expression	Category	Term	Count	%	PValue
Up-regulated	GOTERM_BP_DIRECT	GO:0042787~protein ubiquitination involved in ubiquitin-dependent protein catabolic process	7	0.02	0.01
	GOTERM_BP_DIRECT	GO:0050821~protein stabilization	6	0.01	0.03
	GOTERM_BP_DIRECT	GO:0042493~response to drug	5	0.01	0.03
	GOTERM_BP_DIRECT	GO:0070098~chemokine-mediated signaling pathway	4	0.01	0.04
	GOTERM_BP_DIRECT	GO:0002437~inflammatory response to antigenic stimulus	3	0.01	0.01
	GOTERM_CC_DIRECT	GO:0005737~cytoplasm	62	0.13	<0.01
	GOTERM_CC_DIRECT	GO:0005634~nucleus	56	0.12	0.03
	GOTERM_CC_DIRECT	GO:0070062~extracellular exosome	52	0.11	0.01
	GOTERM_CC_DIRECT	GO:0005654~nucleoplasm	35	0.08	<0.01
	GOTERM_CC_DIRECT	GO:0005730~nucleolus	22	0.05	<0.01
	GOTERM_MF_DIRECT	GO:0044822~poly(A) RNA binding	39	0.08	<0.01
	GOTERM_MF_DIRECT	GO:0005524~ATP binding	30	0.07	0.04
	GOTERM_MF_DIRECT	GO:0000166~nucleotide binding	11	0.02	0.03
	GOTERM_MF_DIRECT	GO:0004672~protein kinase activity	8	0.02	0.02
	GOTERM_MF_DIRECT	GO:0003714~transcription corepressor activity	6	0.01	0.04
Down-regulated	GOTERM_BP_DIRECT	GO:0045087~innate immune response	6	0.29	<0.01
	GOTERM_BP_DIRECT	GO:0006955~immune response	5	0.24	<0.01
	GOTERM_BP_DIRECT	GO:0030593~neutrophil chemotaxis	4	0.20	<0.01
	GOTERM_BP_DIRECT	GO:0071347~cellular response to interleukin-1	4	0.20	<0.01
	GOTERM_BP_DIRECT	GO:0050729~positive regulation of inflammatory response	4	0.20	<0.01
	GOTERM_CC_DIRECT	GO:0005615~extracellular space	10	0.49	<0.01
	GOTERM_CC_DIRECT	GO:0005829~cytosol	10	0.49	<0.01

GOTERM_CC_DIRECT	GO:0005576~extracellular region	8	0.39	<0.01
GOTERM_CC_DIRECT	GO:0070062~extracellular exosome	8	0.39	0.02
GOTERM_CC_DIRECT	GO:0005856~cytoskeleton	3	0.15	0.05
GOTERM_MF_DIRECT	GO:0005515~protein binding	14	0.69	0.04
GOTERM_MF_DIRECT	GO:0035662~Toll-like receptor 4 binding	2	0.10	<0.01
GOTERM_MF_DIRECT	GO:0050544~arachidonic acid binding	2	0.10	0.01
GOTERM_MF_DIRECT	GO:0031726~CCR1 chemokine receptor binding	2	0.10	0.01
GOTERM_MF_DIRECT	GO:0050786~RAGE receptor binding	2	0.10	0.01

Table 2

Gene ontology analysis of differentially expressed genes in transplant patients with AR

Category	Term	Count	%	PValue	Genes
Up-regulated	ptr03013: RNA transport	10	0.02	<0.01	SUMO3, NCBP2, XPO1, NUP153, SUMO1, NUP50, SRRM1, RNPS1, CASC3, PNN
	ptr05203: Viral carcinogenesis	9	0.02	0.05	KRAS, YWHAH, DDX3X, IL6ST, UBE3A, RB1, PMAIP1, RBPJ, DLG1
	ptr01130: Biosynthesis of antibiotics	9	0.02	0.05	ACADM, DLD, TGDS, PFKF, ACLY, ACAT1, OAT, PCCA, PCK1
	ptr04068: FoxO signaling pathway	7	0.02	0.04	GABARAPL1, KRAS, TGFBR2, BNIP3, CCNG2, CHUK, PCK1
	ptr03015: mRNA surveillance pathway	6	0.01	0.04	NCBP2, PPP2CB, SRRM1, RNPS1, CASC3, PNN
Down-regulated	hsa05168: Herpes simplex infection	4	0.20	<0.01	MAPK8, CCL5, TAF6L, HLA-F
	hsa05164: Influenza A	3	0.15	0.02	ACTB, MAPK8, CCL5
	hsa04621: NOD-like receptor signaling pathway	2	0.10	0.04	MAPK8, CCL5
	hsa05416: Viral myocarditis	2	0.10	0.02	ACTB, HLA-F
	hsa05131: Shigellosis	2	0.10	0.03	ACTB, MAPK8

Table 3

KEGG pathway analysis of differentially expressed genes in transplant patients with AR

3.3 KEGG pathway analysis In order to acquire a more detailed information about the essential pathway of those selected DEGs, we also analyzed the most significantly enriched KEGG pathway of the up-regulated and down-regulated DEGs via DAVID, which is uncovered in Table 4 **and** Fig. 3D. The up-regulated DEGs were involved in RNA transport, Viral carcinogenesis, Biosynthesis of antibiotics, The forkhead box O (FoxO) signaling pathway and mRNA surveillance pathway. By contrast, the down-regulated DEGs, namely ACTB, MAPK8, CCL5 and HLA-F were mainly enriched in Herpes simplex infection, Influenza A, NOD-like receptor signaling pathway, Viral myocarditis and Shigellosis.

Gene	Degree of connectivity	P value	LogFC
MAPK8	26	1.25E-02	-1.33
APP	26	1.28E-02	1.32
ACTB	26	1.68E-02	-1.04
HNRNPU	22	2.58E-04	1.07
CYCS	22	1.77E-03	1.16
XPO1	21	2.41E-03	1.06
SRRM1	21	5.11E-04	1.18
SUMO1	21	7.94E-05	1.13
KRAS	21	1.34E-04	1.05
RNPS1	20	6.62E-05	1.01
SNRPD3	18	3.27E-05	1.29
PARP1	18	1.71E-04	1.23
NCBP2	18	6.70E-04	1.12
LUC7L3	17	3.33E-04	1.06
RBM39	17	1.68E-05	1.42
SFPQ	17	4.78E-03	1.10
CCL5	17	2.28E-03	-1.50
HMGB1	17	1.43E-05	1.46

Table 4
Top 18 hub genes with higher degree of connectivity

3.4 Hub genes and Module Screening from PPI Network In order to identify the core genes in those DEGs, we apply the STRING online tool to detect 261 nodes with 845 PPI relationships, which accounted for about 95.7% of these selected DEGs (Fig. 4A). On the basis of the degree of connectivity, we constructed the PPI network and chose the top 18 hub genes (Table 4). The top 18 hub genes with a higher degree of connectivity in AR are as follows: MAPK8, APP, ACTB, HNRNPU, CYCS, XPO1, SRRM1, SUMO1, KRAS, RNPS1, SNRPD3, PARP1, NCBP2, LUC7L3, RBM39, SFPQ, CCL5 and HMGB1. Among these 18 hub genes, MAPK8, ACTB and CCL5 were significantly down-regulated while APP, HNRNPU, CYCS, XPO1, SRRM1, SUMO1, KRAS, RNPS1, SNRPD3, PARP1, NCBP2, LUC7L3, RBM39, SFPQ and HMGB1 were up-regulated. The 18 hub genes could interact with 142 genes directly. Besides, MAPK8, APP and ACTB acted as the most intensive gene, all of which could interact with 23 up-regulated genes and 3 down-regulated genes

respectively. Interestingly, among these hub genes also displayed strong interactions (Fig. 4B). For instance, HMGB1 could directly interact with various genes (ACTB, APP, MAPK8, PARP1, XPO1 and CYCS), and meanwhile XPO1 could interact with 5 genes (HMGB1, SUMO1, SNRPD3, NCBP2 and HNRNPU). Applying the GO function and KEGG pathway analysis of these hub genes, we uncovered MAPK8, XPO1, CYCS and NCBP2 are the 4 high-degree-of- connectivity genes related with RNA transport and immune response. Taken together, these results indicated that these hub genes, especially MAPK8, XPO1, CYCS and HMGB1 may have an essential effect in AR, which are linked with each other tightly (Table 5).

Term	Count	%	PValue	Genes	FDR
cfa05164: Influenza A	5	0.16	<0.01	ACTB, XPO1, CYCS, MAPK8, CCL5	0.26
cfa03013: RNA transport	5	0.16	<0.01	NCBP2, XPO1, SUMO1, SRRM1, RNPS1	0.28
cfa05210: Colorectal cancer	3	0.10	0.01	KRAS, CYCS, MAPK8	7.40
cfa03015: mRNA surveillance pathway	3	0.10	0.01	NCBP2, SRRM1, RNPS1	15.06
cfa03040: Spliceosome	3	0.10	0.03	NCBP2, SNRPD3, HNRNPU	27.65

Table 5
KEGG pathway analysis of top 18 hub genes with higher degree of connectivity

Moreover, we applied MCODE plug-in to reveal the highest modules in the PPI network. We selected the top 3 modules, the scores (Density×#Nodes) of which were ≥ 4. Then, we analyzed the GO function and KEGG pathway enrichment to uncover that Module 1 and Module 3 was associated with RNA transport and mRNA surveillance pathway, while Module 2 was related to inflammatory response and several signaling pathway (Fig. 5 and Table 6).

Module	Term	PValue	FDR	Genes
Module 1	protein ubiquitination involved in ubiquitin-dependent protein catabolic process	<0.01	0.11	CUL2, CUL4A, DZIP3, UBE3A, TRIP12
	RNA transport	<0.01	<0.01	NCBP2, SUMO3, XPO1, SUMO1, NUP153, NUP50, CASC3, PNN
	Ubiquitin mediated proteolysis	<0.01	1.01	CUL2, CUL4A, UBE3A, UBA3, TRIP12
	Nucleotide excision repair	<0.01	0.39	RPA1, ERCC5, CUL4A, GTF2H2
	mRNA surveillance pathway	<0.01	3.06	NCBP2, PPP2CB, CASC3, PNN
Module 2	inflammatory response	<0.01	2.60	HMGB1, PPBP, CCL5, CCL4
	chemokine-mediated signaling pathway	<0.01	1.65	PPBP, CCL5, CCL4
	neutrophil chemotaxis	<0.01	2.76	PPBP, CCL5, CCL4
	Toll-like receptor signaling pathway	<0.01	1.46	MAPK8, CCL5, CCL4, SPP1
	NOD-like receptor signaling pathway	0.01	6.09	MAPK8, BIRC3, CCL5
Module 3	mRNA surveillance pathway	0.01	2.98	SRRM1, RNPS1
	RNA transport	0.02	5.27	SRRM1, RNPS1

Table 6

The enriched pathway of top 3 modules

3.5 Gene Set Enrichment Analysis Furthermore, GSEA was performed to map into GO analysis and KEGG pathway database in order to acquire further insight into the function of the hub gene. According to the cut-off criteria $FDR < 0.05$, $|\text{enrichment score}(ES)| > 0.5$ and gene size ≥ 100 , 6 functional gene sets were enriched totally, which particularly focused on pathways linked with immune response, RNA transport as well as oxidoreductase activity of donors. Six pathway were respectively “myeloid leukocyte mediated immunity”, “regulation of humoral immune response”, “oxidoreductase activity acting on the aldehyde or oxo group of donors”, “mature B cell differentiation involved in immune response”, “regulation of B cell proliferation”, “ncRNA export from nucleus” (Fig. 6).

4. Discussion

In recent years, although the overall incidence and prevalence of acute allograft in kidney transplantation have decreased, it is also a vital reason which leads to graft dysfunction and even death ^[18]. Presently, as

the result of the usage of more effective and advanced immunosuppressive drugs, the incidence of acute rejection in the first year is reduced to approximately 7.9% ^[19]. Currently, renal biopsy remains the gold standard for the specific injury of AR. However, in consideration of its side effects, performing serial renal biopsies are impractical at the same periodicity as blood or urine examination. Besides, Dharnidharka and his colleagues found that cellular and molecular events of AR occur before the rise of the clinical biomarker like serum creatinine as well as according to serum creatinine elevated, clinical doctors can't distinguished a superimposed AR from chronic allograft injury progression, which suggests that functional markers like serum creatinine for glomerular filtration are not as specific as injury markers^[20]. Therefore, it is be in urgent need of more accurate and less invasive methods to diagnose AR so that potent immunosuppressive can be applied in time. Although peripheral blood biomarkers are not as general as that in cardiopathy and tumors due to the complicated molecular mechanisms, it doesn't mean that it makes no sense to perform peripheral blood biomarker to diagnose. Roedder et al. ^[21] used a kidney transplant cohort called the Assessment of Acute Rejection in Renal Transplantation (AART) study to verify a 17-gene signature in the peripheral blood that uncovered an area under curve (AUC) of 0.92 to predict AR. The above studies revealed the potential of peripheral blood biomarkers in diagnosis of AR. In our study, we implemented a synthetic investigation on the expression profiling of PBL from transplant patients with AR which included 6 kidney transplant patients undergoing AR and 9 patients with well-functioning transplant with no clinical evidence of rejection from the GEO database of GSE1563. By the way of defining the absolute value of the logarithm (base 2) fold change (log FC) greater than 2, We identified totally 347 DEGs (account for 2.75% of all genes) where 301 genes were upregulated and 46 were downregulated. In order to have a further detecting and analyzing these DEGs, we performed GO function, KEGG pathway to select 10 sensitive genes and top 18 hub genes were detected via PPI network and connectivity analysis of these DEGs. Additionally, we also applied GSEA to reveal the potential mechanisms of AR through these hub genes.

There were a great many factors related to transplant patients occurring AR, but the primary mechanism to contribute to the AR is still controversial, which is the reason that why it is difficult to diagnose and treat AR. According to our study, we used GO term enrichment and PPI analysis to uncover that the DEGs were closely interrelated with immune response and inflammatory process, especially downregulated DEGs. It is clear that the biological process associated with immunity plays an important role in the development of AR.

On the basis of pathophysiology, AR fall into two categories, which are antibody-mediated rejection (ABMR) and acute T-cell mediated rejection (TCMR) respectively ^[22]. On the one hand, Inflammation of glomeruli and peritubular capillary are always attributed to ABMR. Patients with ABMR usually verifies evidence of circulating donor-specific alloantibodies and immunological evidence of antibody-mediated injury ^[23]. It is reported that acute ABMR occur in about 7% of patients and it can be up to 50% in patients with human leukocyte antigen (HLA)-incompatible transplants ^[24-25]. It is much worse plenty of these patients may develop transplant glomerulopathy or chronic ABMR and have beyond a quadruple risk of graft loss compared to control groups ^[26]. On the other hand, TCMR is characterized by lymphocytic

infiltration of the renal tubules, interstitium and arterial intima ^[27]. Zoghby et al. proved that TCMR is one of the most important and serious complications occurring in the renal transplant patients and it will cause graft loss and permanent impairment of graft function if not early diagnosis ^[28]. Therefore, we found that both ABMR and TCMR are equally essential for renal transplant patients which need to early detection and treatment.

In our GO analysis, it is indicated that DEGs, especially down-regulated DEGs, were mainly associated with immune response as well as neutrophil chemotaxis, innate immune response and inflammatory response. To our knowledge, ABMR is linked with antibodies against foreign donor antigens, especially HLA antigen which contributes to impairing the allograft by the way of activating the complement-dependent pathway and independent mechanisms recruiting NK cells, macrophages, polymorphonuclear cells and platelets to attack the allograft ^[29]. According to this mechanism, we uncovered that there were 3 genes (MAPK8, CCL5 and HMGB1) possibly related to the immune response occurring in ABMR. Haylett et al. discovered that MHC class II molecules can adjust mitogen-activated protein kinase 8 (MAPK8) pathway and the expression of c-Fos in B cells which can induce B cell to release the antibody ^[30]. In another study, MAPK8 was related to the release of BECN1 from its complex with BCL2 apoptosis regulator (BCL2), which in turn results in macrophages, an important class of antigen-presenting cells that activated adaptive immune responses autophagy ^[31]. Besides, it is reported that Fingolimod can increase the C-C motif chemokine ligand 5 (CCL5) in peripheral blood to inhibit the egress of lymphocyte involved in antigen presentation, which restrain B cells for humoral immunity ^[32]. As the same result, Sullivan et al. demonstrated endogenous CCL5 had a neutralization effect and inhibited B cell proliferation and IgM secretion when B cells stimulation ^[33]. As for high mobility group box 1 (HMGB1), through Sprague-Dawley rat hearts transplanted heterotopically into BALB/c mice, it is indicated HMGB1 regulated B cell activation and IFN- γ and IL-17A production which plays an important role in mediating acute xenograft rejection^[34]. Thus, our results suggested the activation and proliferation of B cell and other antigen-presenting cells (APC) attach great importance to the development of ABMR via MAPK8, CCL5 and HMGB1 and further affect the severity of AR.

In TCMR, APC recognize the foreign donor antigens in the transplanted organ via various pathways to activate the recipient's lymphocytes. As a result, T cells were activated and infiltrated which can damage the allograft. Thus, the most important part in TCMR is the activation of T cell. It is reported that some molecules can share the important rejection mechanisms between B cells and NK cells in ABMR and effector T cells in TCMR ^[35]. Therefore, we predicted the mechanisms of T lymphocytes activity and recruitment may also be associated with those 3 hub genes (MAPK8, CCL5 and HMGB1) through GO and KEGG analysis. MAPK8 signaling has been implicated in multiple T cell functions. Similar to MAPK8 activation response in B cells, the MAPK8 is also synergistically activated by co-stimulation of the TCR with antibodies to its CD3 component and the CD28 auxiliary receptor ^[36]. Meanwhile, the activation of MAPK8 in T cells can inhibit the proliferation and infiltration due to neutralization. Additionally, the misbalanced level of chemokines like CCL5 is associated with the activation and function of cytotoxic T lymphocytes and may constrain CD8 + T cell out of lymphoid organs ^[32, 37]. Furthermore, it is reported

that HMGB1 can recruit cells across endothelial barriers and induced the production of tumor-necrosis (TNF) and interferon, which is an nuclear weapon in the immune arsenal ^[38]. In short, from our perspective based on the GO and KEGG analyses, the regulation of immune-related genes involving MAPK8, CCL5 and HMGB1 can play an essential role in ABMR and TCMR which equally have an important effect in the occurrence and progression of AR. Thus, the detection of these target genes in peripheral blood may be a potential candidate for early diagnose of AR.

The PPI network could construct an obvious framework to have a better comprehension of the function of the proteome ^[39]. Through the enriched pathway of top 3 modules, we indicated that the interactions among the proteins in AR are specifically associated with inflammatory response, chemokine-mediated signaling pathway and neutrophil chemotaxis, which is as the same results as above. It emphasizes again that the interaction of immune-related genes can regulate the humoral immunity and cellular immunity associated with AR. Surprisingly, from enriched pathway of Modules 1, we found that some related proteins (NCBP2 and XPO1) in AR are also linked with RNA transport and mRNA surveillance pathway. It implies that RNA transport will be another mechanism of AR development. Coincidentally, we performed GSEA and discovered that non-coding RNA (ncRNA) export from nucleus is one of the key biological processes of AR. We applied DAVID to uncover the function of 18 hub genes and verified the same evidence that nuclear cap binding protein subunit 2 (NCBP2) and exportin 1 (XPO1) have a relationship with RNA transport. Therefore, we speculated that RNA transport can regulate some ncRNA transport which can be detected for diagnosis. Anglicheau et al. demonstrated that the levels of six miRNAs (miR-142-5p, miR-155, miR-223, miR-10b, miR-30a-3p, and let-7c) can become the biomarkers to diagnose AR, among which miR-142-5p, miR-155 and miR-233 can predicted AR with above 90% specificity and sensitivity^[40]. Büssing et al. used *C. elegans* and *Drosophila* to prove that as a nuclear export receptor, XPO1 interacted with NCBP2 which is a subunit of the nuclear cap-binding complex to promote the pri-miRNA to pre-miRNA processing and induce nuclear export of miRNAs ^[41]. The above studies further consolidated our analysis, which revealed that NCBP2 and XPO1 were inextricably linked with the process of ncRNA, especially miRNA transport. Hence, monitoring the ncRNA transport genes and even specific miRNA of AR is of great importance for the diagnosis of AR.

Moreover, when we sifted the GSEA carefully, we uncovered that the development of AR may also be attributed to oxidoreductase activity acting on the aldehyde or oxo group of donors associated with amyloid beta precursor protein (APP). A part of oxidoreductase like Xanthine oxidase can increase the oxidative radical production, which may result in the dysregulation of redox homeostasis and excessive generation of reactive oxygen species (ROS), culminating in oxidative stress and the linked oxidative damage of cellular components ^[42]. Oxidative stress and ROS play an essential role in the pathogenesis of AR. Additionally, oxidative stress can activate autophagy, which can bring about the promotion of inflammatory response indirectly and enhance the progression of AR. APP is an oxidative stress responsive molecule. With APP overexpression, it will damage the structure of mitochondria and increase ROS production, which enhanced oxidative stress ^[43]. Besides, Sennvik et al. used western blotting to compare the cerebrospinal fluid from Alzheimer's disease patients with healthy individual and revealed

APP underwent posttranslational proteolytic processing by α -, β -, γ -secretases, which can produce soluble amyloid protein or APP components with amyloidogenic characters to induce oxidative stress^[44]. It is consistent with our GO and KEGG analyses that protein stabilization is an important process of AR. Therefore, we hypothesized that APP is linked with oxidative stress resulting in protein hydrolysis and inflammatory response. Detecting this gene can early discover the subtle injury of AR in order to deal with it as early as possible.

Conclusively, applying a series of bioinformatics analysis, we provide a comprehensive and novel demonstration of gene expression profiles to recognize DEG expressing in peripheral blood, which may play critical roles in the occurrence and development in renal transplant patients with AR. Genes implicated with immune, inflammation, RNA transport as well as oxidative stress were apparently altered in AR patients, among which MAPK8, CCL5 and HMGB1 play core roles in the immune response of AR including ABMR and TCMR. Additionally, the interaction of NCBP2 and XPO1 will become the new mechanism to speculate the ncRNA transport of AR and may discover more novel miRNA of AR via the mechanism of these two genes. Furthermore, the graft dysfunction and fibrosis were associated with oxidative stress linked with APP in AR, which can be a promising strategy to diagnose and treat AR from another perspective. Taken together, we found that MAPK8, CCL5, HMGB1, NCBP2, XPO1 and APP may be regarded as potential serum biomarkers and targets of therapy. Certainly, we need further experiments and investigations to make sure the correlation of these dysregulated genes in order to allow these biomarkers to be applied more ordinarily in clinic. We expect this kind of analyzing method will provide more valuable and precise information for our future study on the molecular mechanisms of AR and offer more accurate evidences for detection of new diagnosis biomarkers and therapeutic strategies.

Declarations

1. Ethics approval and consent to participate

Not applicable

2. Consent for publication

Not applicable

3. Availability of data and materials

All data generated or analysed during this study are included in this published article

4. Competing interests

The authors declare that they have no competing interests

5. Funding

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6. Authors' contributions

HZ supported the main conception and design work of this manuscript and was a main contributor in writing the manuscript. LL supported a part of design and was a contributor in writing the manuscript. QY contributed some design work and revised the manuscript. All authors read and approved the final manuscript and have agreed both to be personally accountable for their own contributions and ensured that questions related to the accuracy or integrity of any part of the work.

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Figures

Figure 1

Figure 1

Flow diagram of the analysis process data collection, preprocessing, analysis and verification.

Figure 2
A

B

Figure 2

Identification of DEG of PBL in GSE1563. (A) Volcano plot of total 347 DEGs detected in AR from GSE1563. The red dots are on behalf of 301 upregulated DEGs, while green dots are on behalf of 46 downregulated DEGs. The gray dots mean the no differentially expressed genes. (B) Heatmeap of the expression levels of the top 30 upregulated DEGs and top30 downregualted DEGs applying the GEO2R online analysis tool on the basis of $\log_{2}FC \leq -1$ or $\log_{2}FC \geq 1$ and P value < 0.05 criteria.

Figure 3

Figure 3

The scatter plots of GO and KEGG pathway enrichment plot of AR. (A) The GO pathway enrichment scatter plot in the biological process (BP);(B) The GO pathway enrichment scatter plot in the cellular component (CC); (C) The GO pathway enrichment scatter plot in the molecular function (MF); (D) KEGG pathway enrichment scatter plots of AR.

Figure 4

Figure 4

Protein-protein interaction(PPI) network. (A) The PPI network of entire DEGs. (B) The PPI network of top 18 hub genes with high connectivity degree according to the information of the STRING protein query with maximum number of interactors ≤ 5 , confidence score ≥ 0.4 .

Figure 5

Figure 5

The module analysis of AR (A) The PPI network of Module 1 (B) The PPI network of Module 2 (C) The PPI network of Module 3.

Figure 6

Figure 6

Gene set enrichment analysis of AR (A) MAPK8 is negatively correlated with myeloid leukocyte mediated immunity; (B) CCL5 is negatively correlated with regulation of humoral immune response; (C) APP is positively correlated with oxidoreductase activity acting on the aldehyde or oxo group of donors; (D) HMGB1 is negatively correlated with mature B cell differentiation involved in immune response; (E) NCBP2 is negatively correlated with regulation of B cell proliferation; (F) XPO1 is positively correlated with ncRNA export from nucleus”