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Study of regulatory pathway of related molecules in hemolytic uremic syndrome

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Abstract. – OBJECTIVE: To screen Hemolytic Uremic Syndrome (HUS) related differentially expressed genes using the microarray data of neonatal microvascular endothelial cells from human skin treated with or without Shiga toxin, and study the the mechanism of HUS from multiple angles.

MATERIALS AND METHODS: The microarray dataset GSE32710 was download from gene expression database GEO (Gene Expression Omnibus), which included a total of 12 samples, 6 samples were treated with Shiga toxin while the others were normal without any treatments. Then the raw chip data were preprocessed by R package, and T-test was utilized for differentially expressed genes screening. The selected differentially expressed genes were subjected to GO functional and KEGG pathway analysis. Following that, human protein interaction network, synergetic effects among the differentially expressed genes and regulation of post-transcriptional miRNA were integrated so as to construct a molecular interaction network associated with HUS and excavate the sub-function modules.

RESULTS: Trough differential expression screening, 195 of HUS related marker genes were obtained, and 294 of gene pairs with significant co-expression were achieved. Molecular interaction network associated with HUS excavated 302 miRNAs and 117 differentially expressed genes, among which miRNA-30c and miRNA-30d may play important roles during the development of HUS.

CONCLUSIONS: In this study, we used a method that explained the biological mechanism of HUS systematically from gene transcription level and different levels of biological information such as protein interaction, post-transcriptional regulation of gene expression as well as synergy effects of gene expression, which may provide new therapeutic targets for HUS.

Key words:
Hemolytic Uremic Syndrome, Differential expression, Interaction network, Functional pathway enrichment.

Abbreviations

Stx = Shiga toxin; SDF-1 = Stromal cell-derived factor 1; CXCR4 = Chemokine (c-x-c motif) receptor 4; CXCR7 = chemokine receptor 7; GO = Gene Ontology; DAVID = Database for Annotation, Visualization and Integrated Discovery; HPRD = Human Protein Reference Database; 3'UTR = 3'Untranslated Region; p53 = tumor protein 53; MAPK: Mitogen-Activated Protein kinase; ERK = Extracellular signal-regulated kinases; JNK = Jun N-terminal kinases; E1F4E = Datasheets; THP1 = Human monocytic cell line (derived from an acute monocytic leukemia patient).

Introduction

Hemolytic uremic syndrome (HUS) is a disease characterized by non-immune hemolytic anemia, thrombocytopenia and renal impairment. HUS can cause microvascular disease, including vascular wall thickening, endothelial swelling, accumulation of subcutaneous layer of protein and cellular debris, resulting in a serious impact on human health[1]. In children, the disease is often triggered by Shiga toxin (Stx) producing *E. coli* strains, which can bind to the globotriaosylceramide (Gb3) receptor on the surface of endothelial cells, especially in the glomerular microcirculation[2]. After internalization of the toxin, there is retrograde transport to the ribosome, inhibition of protein synthesis, endothelial cell death, and organ hypoperfusion and dysfunction[3,4]. In addition, there is activation of numerous inflammatory cytokines and chemokines that have the potential to cause vascular injury and mediate tissue damage[5].

Although great improvements have been made in the past few years, yet the molecular mechanisms of this disease is still not well understood. Tania et al took advantage of microarray technologies to high-throughput screen the disease-

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related differentially expressed genes (DEGs) and proteins and they found CXCR4/CXCR7/SDF-1 pathway played an important role in Stx-mediated pathogenesis[6,7]. Their studies demonstrated there was marked elevation in blood SDF-1 before the onset of HUS and azotemia, which could contribute to the pathogenesis of HUS. While SDF-1 has been shown to enhance platelet activation by low levels of ADP[8] or thrombin[9], leading to increased aggregation[10] through CXCR4 platelet surface expression. Furthermore, SDF-1 binding to CXCR4 induced permanent internalization of the receptor from the cell surface, so that by the time HUS ensues, SDF-1 might be cleared from the circulation by CXCR4 or its other scavenger receptor, CXCR7. However, the regulation and interaction mechanisms among differential genes have not been investigated. Thus, in the present study, we aimed to further explore the regulation (miRNAs) and interaction mechanisms of differentially expressed genes associated with HUS using the same microarray data and bioinformatics tools. We anticipated that this study may provide new therapeutic targets for HUS.

Materials and Methods

Data Resources and Reprocessing

The chip dataset GSE32710[11] was downloaded from expression database GEO (Gene Expression Omnibus, http://www.ncbi.nlm.nih.gov/ geo/), which included a total of 12 samples, 6 samples were neonatal microvascular endothelial cells from human skin treated by Shiga toxin for 24 h, while the other six samples were normal without any treatments. Platform Information was: [HG-U133A] Affymetrix Human Genome U133A Array. Twelve raw CEL files of samples processed by chip, as well as the probe annotation file information corresponding to the platform were downloaded. Affy package in Bioconductor of R language was used to preprocess the raw microarray data, including probe mapping and standardization of signal values. If a number of probe sets corresponding to the same gene, then the intermediate value of probe set was considered as the expression level of this gene, while a plurality of genes mapped to the same probe, the probe was thought to have no specificity and would be removed in the subsequent analysis.

Differential Expression Analysis

In order to explore the significantly expressed genes during the development of HUS, differential expression analysis was then performed. Both the sample data treated by Stx and the control samples were subjected to *t*-test. Genes with fold change greater than 2 or less than 0.5, and *p*-value < 0.05 was considered as differentially expresssed genes.

Analysis of Synergetic Effects of Differential Genes

As we know that molecules in vivo would not exercise function independently, especially in complex diseases, so after treatment by Stx, the HUS related genes should also show a synergistic mode of action. For the significant differentially expressed gene sets screened in *t*-test, the co-expression relationship among genes in all samples was calculated, and Pearson correlation coefficient (correlation analysis utilized in R cor function) was selected as a metric standard. The gene pairs that played synergetic roles in the two sample groups respectively were screened (the Pearson correlation coefficient was greater than 0.95 and the significant *p*-value was less than 0.05). In addition, a collection of differential genes with co-expression relations were identified too.

Functional Pathway Annotation of HUS Related Genes

We[12-13] carried out GO function[14] and KEGG (Kyoto Encyclopedia of Genes and Genomes) pathways[15] annotation for the genes showing significant differences in HUS by DAVID[16]. Through GO function enrichment analysis, the HUS related genes were enriched respectively and information about biological processes, cell constitution and molecular functions was obtained. The count value greater than 2 and *p*-value less than 0.05 was taken as the threshold for the screening of significant functional annotation result.

miRNA and Network Analysis

As realization of miRNA function depends on the target genes it binding to, for significantly differentially expressed genes identified in this study, based on sequence matching between miRNA and its target gene, conservatism and structure proximity, miRNA target gene prediction algorithm was carried out to predict the miRNA involved in the development of HUS. In order to improve prediction accuracy, and reduce

false positivity, seven different kinds of miRNA target gene prediction algorithms (PicTar[17], DIANA-microT[18], miRanda[19], miRBase[20], RNAhybrid[21], RNA22[22], TargetScan[23] were utilized to obtain miRNAs. The miRNA predicted by at least three algorithms was considered as the final HUS related miRNAs.

After that, we further did network analysis. As the protein in cells did not function independently, any biological process was completed by a plurality of protein molecules together with other molecules, cooperating with each other. Studying the interaction of proteins was of great significance to understand regulation of biomolecules, transduction of cell signal and metabolic pathways. HPRD database (http://www.hprd.org/)[24] contains information about human proteins, including protein annotation information, protein-protein interaction relations *in vivo*, *vitro* or by yeast two-hybrid assay, modification information of genes after transcription. We got 39240 proteins relations among 9617 proteins from this database.

Similarly, miRNA was another important kind of regulation factor, which could regulate gene expression at post-transcriptional level trough base pairing with 3'UTR of the target gene, inhibiting translation or degradation of mRNA mechanism. We integrated protein interaction and synergetic effects related to differential genes, miRNA regulation so as to construct a HUS related network, and did structure analysis for the network. Finally, the network function module was explored.

Results

Differential Expression Analysis

Differential expression analysis of the standardized data was done after the gene expression chip was preprocessed. At last, 195 differentially expressed genes were screened by *t*-test analysis, among which 53 genes were up-regulated while 142 genes were down-regulated.

Synergetic Effects of Differentially Expressed Genes and miRNA Prediction

Pearson correlation coefficient was taken to analyze co-expression patterns of the genes with significant differences identified in this study. As expected, a total of 294 pairs of gene pairs with significant co-expression were achieved based on the threshold of Pearson correlation coefficient greater than 0.95 and *p*-value less than 0.05. miRNA-target interaction selected by at least three algorithms were taken as reliable HUS related regulation, and combined with the differentially expressed genes the role of miRNA played in HUS was also predicted. Finally, 689 regulating relations after transcription between 303 miRNAs and 101 target genes were obtained.

Functional Annotation of HUS-Related Genes

In order to reveal the mechanism of differentially expressed genes in HUS, the differentially expressed genes obtained in our study were subjected to do GO and KEGG pathway annotation features. GO enrichment analysis included biological processes, cells constitution and molecular functions (Table I). Based on significant level ($p = 0.001$) and parameters (enrichment score 2), through KEGG pathway enrichment analysis, we found the differential genes associated with HUS participated in some cancer-related pathways, such as P53 pathway, MAPK pathway, cytokines interaction and the like (Table II).

Network Analysis and Excavation of Functional Modules

A HUS related molecular interaction network was constructed successfully, as shown in Figure 1. A total of 419 nodes and 1001 molecular interaction (synergistic co-expression, miRNA regulation after transcription and protein interactions) were included in this network, and 302 miRNAs and 117 differentially expressed genes were also contained. HUS regulatory network related genes significantly enriched 46 biological processes, such as cell apoptosis, cell death, intracellular molecular synthesis as well as cell metabolism and other processes (Table III).

The cutoff of degree for nodes was set at 2 based on the software Mcode[25] in Cytoscape[26], and four modules in HUS specific system regulatory network were identified, as shown in Figure 2. The first module included 16 genes with significant differential expression by functional annotation, and we found that these genes were involved in cellular RNA process, intracellular signaling cascades and cell cycle regulation (Table IV); In module 2, there were 8 closely linked genes and they were involved in cell proliferation, differentiation and metabolic processes; while in module 4, 2 miRNAs (miRNA30-c and miRNA30-d) regulated 2 abnormal genes (Table IV).

Table I. The top 10 GO biological processes, cellular components and molecular function enriched by hemolytic uremic syndrome related genes.

GO enrichment	Term	Count	*p*-value	Description
Biological processes	GO:0009611	20	1.61E-05	Response to wounding
	GO:0006357	22	1.33E-04	Regulation of transcription from RNA polymerase II promoter
	GO:0010035	11	1.62E-04	Response to inorganic substance
	GO:0010033	21	3.30E-04	Response to organic substance
	GO:0007610	16	4.49E-04	Behavior
	GO:0006954	13	4.85E-04	Inflammatory response
	GO:0014070	8	5.80E-04	Response to organic cyclic substance
	GO:0016265	20	9.16E-04	Death
	GO:0006952	18	9.80E-04	Defense response
	GO:0042493	10	0.001074	Response to drug
Cellular components	GO:0005615	17	0.002507	Extracellular space
	GO:0005730	17	0.003021	Nucleolus
	GO:0031981	26	0.009921	Nuclear lumen
	GO:0044421	19	0.013103	Extracellular region part
	GO:0043233	30	0.015453	Organelle lumen
	GO:0031974	30	0.019772	Membrane-enclosed lumen
	GO:0070013	29	0.020196	Intracellular organelle lumen
	GO:0030672	3	0.024569	Synaptic vesicle membrane
	GO:0005886	52	0.027519	Plasma membrane
	GO:0043232	38	0.031694	Intracellular non-membrane-bounded organelle
Molecular function	GO:0003700	26	4.52E-05	Transcription factor activity
	GO:0043565	18	3.10E-04	Sequence-specific DNA binding
	GO:0033549	4	3.31E-04	MAP kinase phosphatase activity
	GO:0017017	4	3.31E-04	MAP kinase tyrosine/serine/threonine phosphatase activity
	GO:0008138	5	0.001292	Protein tyrosine/serine/threonine phosphatase activity
	GO:0030528	30	0.001539	Transcription regulator activity
	GO:0015300	5	0.002768	Solute: solute antiporter activity
	GO:0015298	4	0.004108	Solute:cation antiporter activity
	GO:0005125	8	0.005394	Cytokine activity
	GO:0015297	5	0.006344	Antiporter activity

Discussion

Microarray technology could provide a large number of differentially expressed genes, which provides a wealth of valuable information for us to study the pathogenesis of HUS. In this study, we made use of microarray technology to research the samples treated by Stx for 24 hours and control samples so as to identify the differential genes. Then miRNA prediction and synergetic expression analysis were carried out for the differentially expressed genes. We integrated human protein interaction network to build interaction network for HUS related molecules. The sub-modules in the network were excavated simultaneously. Finally, differentially expressed genes were subjected to functional and pathway enrichment analysis and their biological mechanisms were further demonstrated.

Numerous reports demonstrated that Shiga-like toxin generated from *E. coli* was a common cause of HUS. Diana reported that Shiga-like toxin could inhibit protein synthesis in the host, leading to cell death *in vivo*[27], which was consistent with our findings HUS related differential genes were involved in molecular synthesis and affected programmed cell death.

Table II. KEGG pathway enriched by hemolytic uremic syndrome related genes.

Count	*p*-value	Description	
hsa04010	10	0.005744075	MAPK signaling pathway
hsa04115	5	0.010179857	p53 signaling pathway
hsa04060	9	0.016104474	Cytokine-cytokine receptor interaction
hsa05200	10	0.020495775	Pathways in cancer

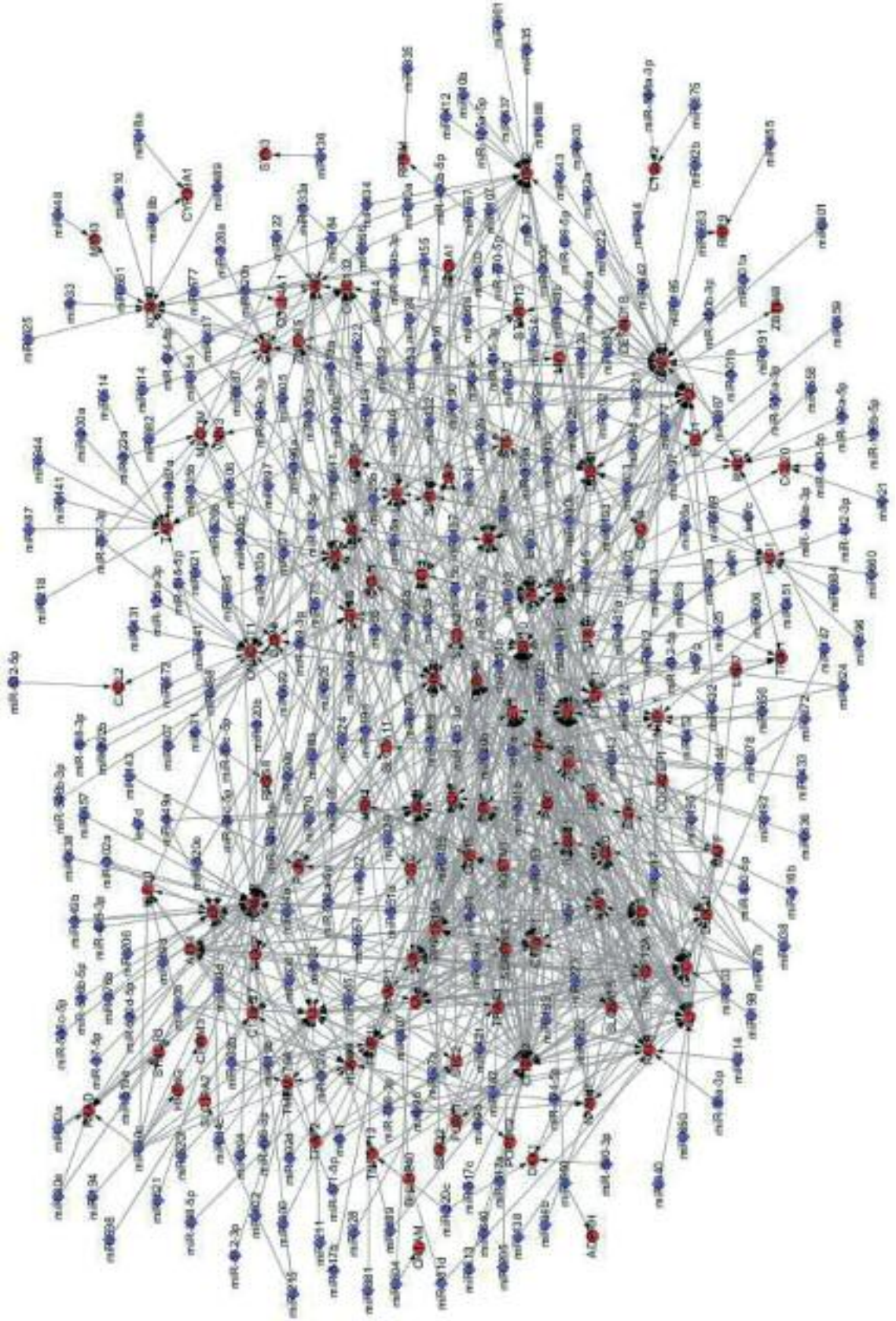

Figure 1. The interaction network of hemolytic uremic syndrome related molecule.

Table III. The top 10 functional annotation of hemolytic uremic syndrome regulatory network related genes.

KEGG	Count	*p*-value	Description
GO:0010942	14	0.001986028	Positive regulation of cell death
GO:0043068	14	0.001907236	Positive regulation of programmed cell death
GO:0043065	14	0.001793913	Positive regulation of apoptosis
GO:0006915	17	0.002065357	Apoptosis
GO:0012501	17	0.002396653	Programmed cell death
GO:0016265	20	9.16E-04	Death
GO:0008219	19	0.002112333	Cell death
GO:0010941	20	0.003525333	Regulation of cell death
GO:0043067	20	0.003386268	Regulation of programmed cell death
GO:0042981	20	0.003037699	Regulation of apoptosis

Through KEGG pathway enrichment analysis we found that the differential genes associated with HUS participated in mitogen-activated protein kinase (MAPK) pathway. The MAPK pathways consist of a series of kinases, which sequentially phosphorylate downstream kinases to transmit intracellular signals. These pathways typically involve the activation of a MAP kinase kinase kinase (MAPKKK), which phosphorylates and activates a MAP kinase kinase (MAPKK), which in turn phosphorylates a MAPK. Once activated, the MAPKs phosphorylate down-stream cellular substrates such as transcription factors and other protein kinases[28]. A recent research about ricin on health showed MAPK played an important role in the regulation of response to pro-inflammatory gene activation, ricin could activate ERK, JNK and MAPK genes in mouse kidney, and up-regulated TNF-α (tumor necrosis factor α), interleukin (IL)-1β, and IL-6, leading to HUS symptoms like microangiopathy, hemolytic anemia, thrombocytopenia, acute renal exhaustion at different levels in mice[29]. Another study about the impact of Stx on MAPK pathway and elF4E also demonstrated that Stx could affect activation of multiple intracellular genes such as MAPK and so on, leading to significant changes of TNF, IL-8, etc. at multi-

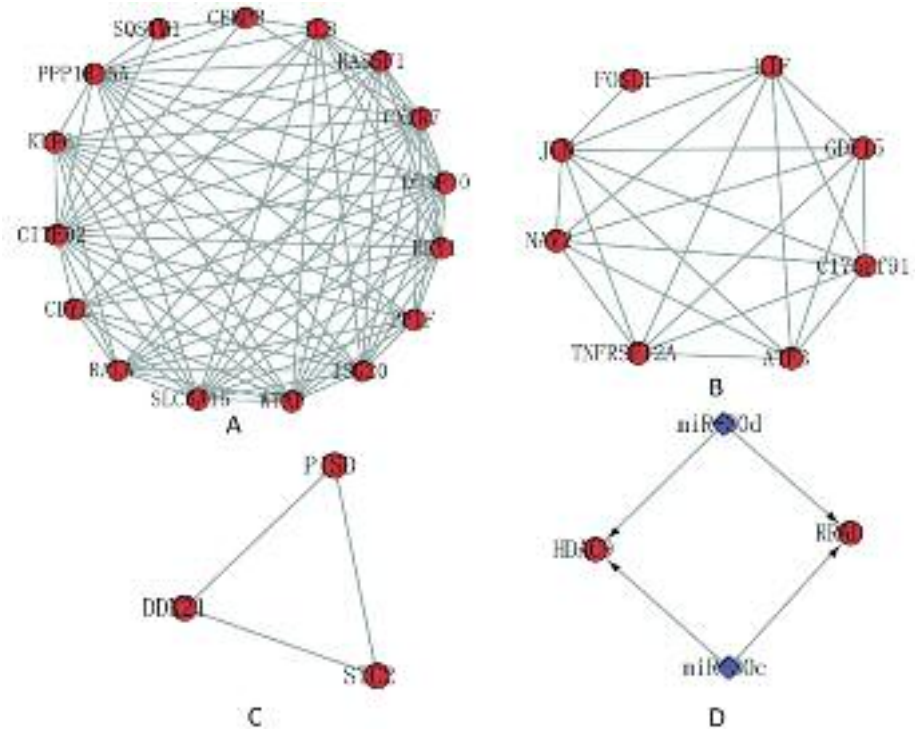

Figure 2. Modules in specific systematic regulation networks of hemolytic uremic syndrome.

Table IV. Functional annotation of genes in the first and second module.

Module	Term	Count	*p*-value	Description
Module 1	GO:0007050	3	0.005650843	Cell cycle arrest
	GO:0006357	5	0.007016258	Regulation of transcription from RNA polymerase II promoter
	GO:0045944	4	0.007283941	Positive regulation of transcription from RNA polymerase II promoter
	GO:0045893	4	0.014447265	Positive regulation of transcription, DNA-dependent
	GO:0051254	4	0.014774943	Positive regulation of RNA metabolic process
	GO:0006916	3	0.021268763	Anti-apoptosis
	GO:0048514	3	0.022245335	Blood vessel morphogenesis
	GO:0045941	4	0.022552414	Positive regulation of transcription
	GO:0010628	4	0.024378494	Positive regulation of gene expression
	GO:0001892	2	0.026296962	Embryonic placenta development
Module 2	GO:0008284	3	0.005651	Positive regulation of cell proliferation
	GO:0042127	5	0.007016	Regulation of cell proliferation
	GO:0045597	4	0.007284	Positive regulation of cell differentiation
	GO:0051094	4	0.014447	Positive regulation of developmental process
	GO:0051726	4	0.014775	Regulation of cell cycle
	GO:0007167	3	0.021269	Enzyme linked receptor protein signaling pathway
	GO:0002763	3	0.022245	Positive regulation of myeloid leukocyte differentiation
	GO:0045944	4	0.022552	Positive regulation of transcription from RNA polymerase II promoter
	GO:0045893	4	0.024378	Positive regulation of transcription, DNA-dependent
	GO:0051254	2	0.026297	Positive regulation of RNA metabolic process

ple levels, which may cause HUS[30]. In studies measuring MAPK activation in differentiated, macrophage-like THP-1 cells, it was noted that a p38 MAPK inhibitor partially blocked soluble TNF-α production induced by Stx[31]. Furthermore, Stx could induce the prolonged expression or release of cytokines, which is in part a result of the stabilization of cytokine and chemokine mRNA transcripts[32]. The prolonged activation of MAPK cascades by Stx may be critical for transcript stabilization and prolonged expression of cytokines. The consequences of prolonged activation of MAPKs by Shiga toxin1 in THP-1 cells may be correlated with the prolonged activation of cytokine genes and/or the stabilization of cytokine mRNA transcripts, resulting in increased levels of IL-1 and IL-8[29].

It was reported that chemokine MCP-1 (membrane cofactor protein-1) and IL-8 were closely related with pathogenesis of HUS. In children patients with HUS, MCP-1 and IL-8 level in urine were seriously excessive. Furthermore, they presented apparent positive correlation, yet no clear correlation showed in serum. Through living tissue slicing studies, both the chemokines could respectively supplement and activate mononuclear (MO) and polymorphonuclear cells (PMNs), while MOs and PMNs played an important role in glomerular injury[33]. Earlier study also showed that IL-8 exerted a key role in the development mechanism of HUS[34]. In addition, children with HUS had prostacyclin metabolic abnormalities, and low level activity of prostacyclin was hereditary[35], which indicated cell metabolism existed in the process of uremia. Although the chemokines played great important roles in the pathogenesis of HUS, yet their precise roles required additional study.

Conclusions

In the molecular interaction network we constructed, a large number of miRNA were predicted, they may regulate their target genes by acting on the development and progression of uremia. For example, Lorenzen et al[36] reported miR-24 and miR-126 levels were much higher in Shiga toxin-induced uremic patients than normal population. Studying inpatients and subsequent tracking survey, miR-24 together with miR-126 showed a close relationship with patients' neurological symptoms and platelet number. Although there was nearly no report about miR-30 in HUS, yet results from cancer research found miR-30 family members play important roles in tumor apoptosis[37,38]. While apoptosis was demonstrated in kidneys from patients with HUS and in mice

inoculated with Stx-positive strains[27]. The miR-30c and miR-30d reported in this study may be considered as the new therapy targets for HUS. However, studying disease according to gene expression in a multi-omics layer, which contributed to a more profound understanding of pathogenesis of disease, and also had a positive effect on the future prevention, therapy of disease and drug targets mining.

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Conflict of Interest

The Authors declare that there are no conflicts of interest.

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