ORIGINAL ARTICLE

Bioinformatic analysis of differentially expressed genes involved in the hepatitis B virus-associated acute liver failure

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Abstract

Background **: The rarity of acute liver failure, along with its severity and heterogeneity, has resulted in a very limited evidence to understand of the molecular mechanism. To analyze the differentially expressed genes (DEGs) in the Hepatitis B Virus -Associated Acute Liver Failure and elucidate the biological significance of the DEGs.**

Methods **: Firstly, differentially expressed genes (DEGs) between seventeen HBV-associated acute liver failure liver samples and ten control normal liver samples were identified by R package. Then, the enriched GO terms and KEGG pathways of those DEGs were obtained using the Database for Annotation, Visualization and Integrated Discovery (DAVID). Finally, protein-protein interactions (PPI) network of those DEGs were constructed using STRING database and visualized by Cytoscape software.**

Results **: A total of 328 DEGs were identified in Hepatitis B Virus-Associated Acute Liver Failure group compared with the control group. Several novel biomarkers that might play important roles in HBV-associated acute liver failure were identified through the analysis of gene microarray in GEO. Furthermore, DEGs with high connectivity degrees, such as KNG1, PLG, F2 and pathways such as complement and coagulation cascades were noticed.**

Conclusion **: DEGs with high connectivity degrees, such as KNG1, PLG and their relative pathway complement and coagulation cascades may be important for further understanding of the molecular mechanism of HBV-associated acute liver failure.** (Acta gastroenterol. belg., **2018**, 81, **288-294**).

Keywords : HBV-associated acute liver failure, Differentially expressed genes, Protein-protein interactions, Bioinformatic.

Abbreviations : DEGs, differentially expressed genes ; PPI, protein-protein interactions ; HSC, hepatic stellate cell ; HSPC, hepatic stellate progenitor cell ; GEO, Gene Expression Omnibus ; HBV, Hepatitis B Virus ; DAVID, Database for Annotation, Visualization and Integrated Discovery ; GO, Gene Ontology ; KEGG, Kyoto Encyclopedia of Genes and Genomes ; ALB, albumin ; C5a, complement component 5 ; C1s, complement component 1 ; ApoH, apolipoprotein H ; ApoA1, Apolipoprotein A1 ; PPARs, Peroxisome proliferator-activated receptors ; HNA2, human nonmercaptalbumin-2 ; KNG1, Kininogen-1 ; PLG, plasminogen ; F2, Coagulation factor II ; PT, prothrombin time.

Introduction

Acute liver failure is a rare but life-threatening critical illness that occurs most often in patients who do not have preexisting liver disease. With an incidence of fewer than 10 cases per million persons per year in the developed world (1). Hepatitis B virus (HBV) is a major cause of ALF worldwide : ≈1% of acute hepatitis B patients develop fulminant hepatitis (2). However, the rarity of acute liver failure, along with its severity and heterogeneity, has resulted in a very limited evidence to understand the pathogenesis. Various molecular mechanism and different biomarkers related to HBV-associated acute liver failure have been identified. Nissim et al. (3) have inferred that HBV-related ALF is associated with expression of genes related to HSPC, hepatic stellate cell (HSC) activation and fibrogenesis, along with an overriding cell proliferation and tumorigenesis gene signature. Farci et al. (4) indicated that HBcAg is the target of a germline human V_H gene. These data suggest that humoral immunity may exert a primary role in the pathogenesis of HBV-associated ALF. Barthel et al. (5) described a pathomechanism in HBV expressing hepatocytesm where the insulin receptor is intracellularly retained, preventing compensatory liver regeneration that finally could foster chronic liver damage and liver disease progression. Despite a great number of previous researches, molecular mechanism of HBV-associated acute liver failure has not been fully grasped. Hence, further researches, are still needed to find out new molecular mechanism or biomarkers in an effort to improve the prognosis, diagnosis and treatment of HBV-associated acute liver failure. In this study, by analyzing the gene expression microarray of HBV-associated acute liver failure in GEO database, we further study the molecular mechanism and some biomarkers of HBV-associated acute liver failure. In general, our finding can promote our understanding of HBV-associated acute liver failure.

Materials and methods

Characteristics of patients

Four patients with HBV-associated acute liver failure were previously healthy, young adult individuals who

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Table 1. — **Results of laboratory tests in four patients with HBV-associated acute liver failure**

	Massive Hepatic Necrosis (MHN)				Submassive Hepatic Necrosis (MHN)			
	Patient 241		Patient 31		Patient 219		Patient 32	
Variable	On Admission	Before OLT	On Admission	Before OLT	On Admission	Before OLT	On Admission	Before OLT
Platelet count (per mm3)	90,000	93,000	119,000	196,000	192,000	91,000	109,000	141,000
Creatinine (mg/dL)	0.6	1	0.9	0.8	2.6	3.7	1	1
Prothrombin time (INR)*	11	4.5	6.7	4.6	11.5	4.6	4.4	2.5
Bilirubin (mg/dL)								
Total	9.8	20.6	8.9	10.8	5.2	10.4	11.7	13.6
Conjugated		2.2	4.3	2.3	3.2	4.5	10.2	6.9
Aspartate aminotransferase (U/L)	4,000	34	9,793	924	4,565	194	6,544	1,114
Alanine aminotransferase (U/L)	5,700	252	7,573	2,537	4,710	762	11,630	2,402
Alkaline phosphatase (U/L)		167	542	401	326	291	296	290
Lactate dehydrogenase (U/L)	1,460	603	4,972	449	1,411	822	6,481	846
γ-Glutamyltransferase (U/L)		27	80	47	39	36	165	109
Alphafetoprotein (ng/mL)**		2.5		1.21		3.5		105.5
Serology for viral hepatitis A, C, D	Negative		Negative		Negative		Negative	
HBsAg	Positive	Negative	Positive	Positive	Positive	NA	Borderline	Negative
Concentration (µg/mL)				1.74				
Anti-HBs (mIU/mL)	0.6	67.7	0	0	15.3		5.9	8
Anti-HBc	Positive	Positive	Positive	Positive	Positive	NA	Positive	Positive
IgM anti-HBc	Positive	Positive	Positive	Positive	Positive	NA	Positive	Positive
HBeAg	NA	Negative	Negative	Negative	Positive	NA	Negative	Negative
Anti-HBe	NA	Positive	Borderline	Positive	Positive	NA	Positive	Positive
HBV DNA (copies/mL)	760	291		218,000	19,284		185,000	7,500
HDV RNA	Negative		Negative		Negative		Negative	
HCV RNA	Negative		Negative		Negative		Negative	

To convert the values for creatinine to µmol/L, multiply by 88.4 ; to convert the values for total and conjugated bilirubin to µmol/L, multiply by 17.1. ; *Normal range, 0.80 to 1.20 international normalized ratio (INR). ; **Normal range, <10.0 ng/mL. NA denotes not available. ; OLT : orthotopic liver transplantation

suddenly developed acute liver failure. The 4 patients, two males and two females, had a mean age ±SD of 42.0±7 years, none of them had a history of intravenous drug addiction, alcohol abuse or tattooing. All patients suddenly developed all or some of the following symptoms, including fever, abdominal pain, vomiting, nausea, and increasing malaise, and at admission to the hospital all were jaundiced. On examination, there was no evidence of chronic liver disease, such as clubbing, spider angiomas, and splenomegaly. Liver and serum specimens along with the clinical data were received under code to protect the identity of the subjects. The clinical, biochemical, and virologic course was similar in the 4 patients (Table1). The control group was composed of 10 liver donors, 5 females and 5 males, with a mean age ±SD of 35.0±13 years. None of them had evidence of active infection with hepatitis A, B, C, and D as well as for other known viral infections that were analyzed as part of the mandatory liver donor screening. Four cases had no serological evidence of prior exposure to HBV infection, four had isolated anti-HBs and two had both anti-HBs and anti-HBc. The results of liver enzyme levels, HBV serology and liver histology of 8 out of 10 liver donors have been previously reported (6).The control livers were obtained from 10 liver donors during resection for liver angioma. Written informed consent was obtained from each patient or the next of kin. The study was approved by the Office of Human Subjects Research of the National Institutes of Health, granted on the condition that all samples be made anonymous (7).

Gene Expression Microarray Data

In this study, the gene expression microarray data set GSE38941 was downloaded from the Gene Expression Omnibus (GEO, http://www.ncbi.nlm.nih.gov/geo/). GSE38941 (7) is a gene expression profile data including seventeen HBV-associated acute liver failure liver samples and ten control normal liver samples. The platform of this microarray data is GPL570 [HG-U133_Plus_2] Affymetrix Human Genome U133 Plus 2.0 Array.

Preprocessing of the Microarray Data

In the preprocessing stage, we filtered out the unwanted noise of the raw microarray data. The background correction and normalization of raw data was conducted via affy (8) package in R. Moreover, multiple probes that corresponded to one gene symbol were summarized–taking the average expression values of those probes as the expression value of this gene.

Get the Differentially Expressed Genes

After the preprocessing, the first step was to get the differentially expressed genes (DEGs) between the case samples and the control samples. The tool used in this study was the limma (9) package in R. The t-test was conducted on the gene expression values between case samples and control samples and the genes with P value<0.05 and $|\log_2(\text{fold change})|>2$ were selected. The heatmap of the DEGs was obtained through gplots package in R to visualize their expression value in different samples.

GO Enrichment and KEGG Pathway Analysis of the DEGs

GO enrichment and KEGG pathway analysis of the DEGs were conducted after getting the DEGs. Here, DAVID (http://david.abcc.ncifcrf.gov/), Database for

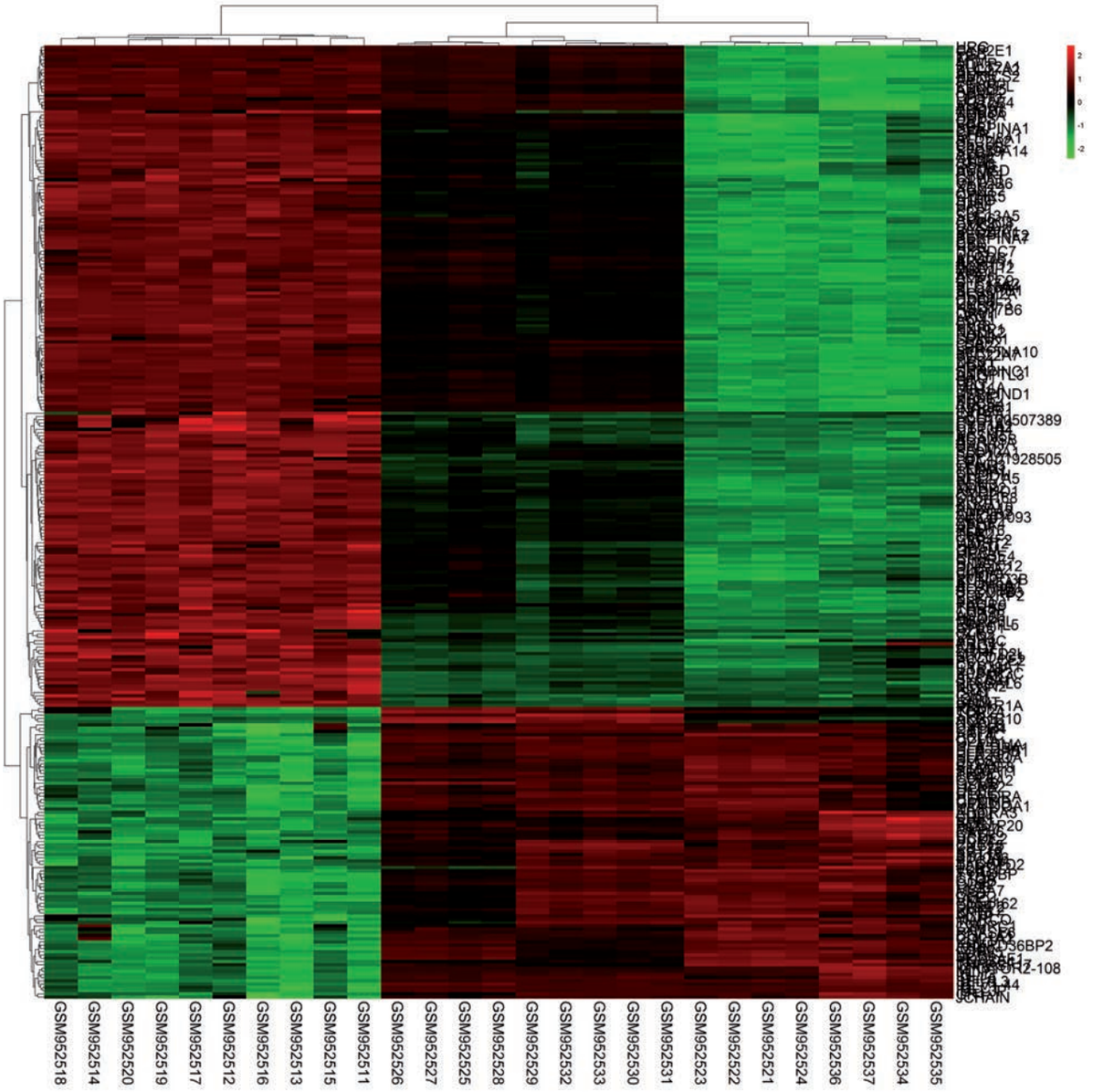

Figure 1. — The heatmap of the DEGs. In the figure 'GSM9525n (n=11, 12, 13, 14, 15, 16, 17, 18, 19, 20)' is the control samples; 'GSM9525n (n=21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37)' is the HBV-associated acute liver failure samples

Annotation, Visualization and Integrated Discovery, was used in this study. It could be used to do functional annotation for a list of genes, gene functional classfication or gene ID conversion. In this study, the module used in this study was the functional annotation. First, we submitted the DEGs list into the database and selected homo sapiens in species column. Finally, the GO terms and the KEGG pathways with P value smaller than 0.05 and at least five genes were selected out as the enriched function of DEGs.

Construct the PPI Network of DEGs

To further investigate the molecular mechanism of HBV-associated acute liver failure, PPI network of the DEGs was constructed through STRING database (http://www.string-db.org/). STRING is an online database which includes experimental as well as predicted interaction information and comprises >1,100 completely sequenced organisms (10). Also, it has a unique scoring framework which assigns the interaction an integrated score to represent its confidence through combining the score of the different sources. Here, we selected the gene-gene interactions, whose integrated scores were bigger than 0.4 (the default threshold in the STRING database), to construct the PPI network and Cytoscape (11) was used for visualization.

Select the Core Gene in the Network

To select core genes (the genes that might be more likely involved in HBV-associated acute liver failure) from PPI network, we analyzed the topological structure of the network and obtained the degree (the number of genes that directly interact with the gene) of each gene. Here, we selected the genes whose degree is beyond 10 as the core genes in the network.

Results

Differentially Expressed Genes (DEGs)

All the four patients with HBV developed progressive encephalopathy, reaching coma grade IV and underwent orthotopic liver transplantation within 8 days from the onset of symptoms. There were 328 DEGs between the case samples and control samples, including 207 up-regulated and 121 down-regulated genes. From the heatmap (Fig. 1), we could get that the gene expression of HBV-associated acute liver failure samples were distinguished from the control samples. But we didn't find any differences between 2 groups in terms of gene signatures.

Enriched GO Terms and KEGG Pathways of DEGs

In this study, a total of 119 enriched GO terms and 43 KEGG pathways were obtained. The top 10 enriched GO terms of the DEGs were shown in Table 2, which were sorted by P value in ascending. Table 2 indicated that the main enriched GO terms was the biological process of cell, such as complement activation, classical complement activation pathway, alternative complement activation pathway, platelet degranulation.

The top 20 enriched KEGG pathways of the DEGs were shown in Table 3, which were sorted by P value in ascending. A few enriched KEGG pathways were directly related to immunity, such as Complement and coagulation cascades, Intestinal immune network for IgA production, Chemical carcinogenesis. What's more, it was possible that other pathways had an important influence on the progression of HBV-associated acute liver failure via some biological process, such as PPAR signaling pathway, Graft-versus-host disease, Glycine, serine and threonine metabolism and etc. ; The KEGG pathways and their corresponding gene number were shown in Fig. 2.

PPI Network of the DEGs and Core Genes in the PPI Network

The PPI (Fig. 3) network contained 185 nodes and 692 edges. The nodes represented the DEGs and the edges represented the interactions between the DEGs. A great number of genes of higher degree, which were the core genes in the PPI network, might relate to HBV-associated acute liver failure more closely. The core genes and their

Table 2. — **The GO terms enriched in DEGs**

Category	GOID	GO term	Count	PValue
CC	GO:0072562	blood microparticle	36	2.80E-31
CC	GO:0005576	extracellular region	93	4.50E-30
CC	GO:0005615	extracellular space	84	3.98E-29
CC	GO:0070062	extracellular exosome	115	8.68E-25
BP	GO:0006958	complement activation, classical pathway	19	2.02E-14
BP	GO:0002576	platelet degranulation	18	5.74E-13
BP	GO:0006957	complement activation, alternative pathway	9	4.56E-12
BP	GO:0006956	complement activation	16	7.08E-12
CC	GO:0031093	platelet alpha granule lumen	13	3.47E-11
MF	GO:0004252	serine-type endopeptidase activity	23	5.56E-11

Table 3. — **The KEGG pathways enriched in DEGs**

Pathway term	Count	PValue
hsa04610:Complement and coagulation cascades	31	1.12E-30
hsa05150:Staphylococcus aureus infection	16	2.09E-12
hsa01100:Metabolic pathways	67	1.47E-10
hsa00830:Retinol metabolism	15	4.87E-10
hsa00982:Drug metabolism - cytochrome P450	14	9.82E-09
hsa04976:Bile secretion	13	1.13E-07
hsa00980:Metabolism of xenobiotics by cytochrome P450	13	2.51E-07
hsa05204:Chemical carcinogenesis	13	6.04E-07
hsa05020:Prion diseases	9	1.12E-06
hsa05322:Systemic lupus erythematosus	15	6.01E-06
hsa05133:Pertussis	11	1.59E-05
hsa05323:Rheumatoid arthritis	11	6.54E-05
hsa05332:Graft-versus-host disease	7	1.47E-04
hsa04672:Intestinal immune network for IgA production	8	1.53E-04
hsa05330:Allograft rejection	7	2.84E-04
hsa00650:Butanoate metabolism	6	4.92E-04
hsa04940:Type I diabetes mellitus	7	5.78E-04
hsa00140:Steroid hormone biosynthesis	8	5.79E-04
hsa00120:Primary bile acid biosynthesis	5	7.15E-04
hsa05310:Asthma	6	8.17E-04

Table 4. — **The core genes and their corresponding degree**

Gene	Degree*	Gene	Degree*
ALB	72	AR	14
KNG1	31	CYP2C9	14
PLG	30	AOX1	14
F2	29	CYP1A1	14
APOA1	26	CYP1A2	14
HSD17B6	25	LPA	14
SERPINC1	25	ABCB4	13
TF	24	SLCO1B3	13
IL8	23	SLC10A1	13
APOB	22	SULT2A1	13
CYP7A1	21	CP	13
CYP2B6	18	CPB2	13
CYP2E1	18	ABCG5	12
AHSG	17	APOH	12
UGT2B4	17	CYP2C8	12
CYP3A4	17	FGB	12
IGF1	17	SERPIND1	12
HPX	17	F9	12
HRG	17	CXCR4	11
SERPINF2	17	PLTP	11
SLCO1B1	16	TOP2A	10
FGA	16	C1S	10
NR1I3	15	CCL5	10
C5	15	F11	10
ITIH4	14		

*The number of genes that directly interact with the genes in the PPI network

corresponding degree were shown in Table 4. There were 11 genes whose degree was beyond 20.

Discussion

Although researchers have made considerable efforts in elucidating the mechanisms of HBV-associated acute liver failure, the pathogenesis of HBV-associated acute liver failure remains unclear. In this research, the genome-wide gene expression analysis was conducted by a high throughput method to identify the DEGs from HBV-associated acute liver failure compared with normal liver tissues. Here, a total number of 328 DEGs from original dataset were identified, including 207 up-regulated and 121 down-regulated genes.

GO analyses revealed that the significant ontology categories included complement activation, classical complement activation pathway, alternative complement activation pathway, platelet degranulation and so on. In the complement activation, for example, Lei et al. (12) found that acute liver failure strongly activates the complement system, leading to a C5a increase. It also implies that the excessive activation of complement, particularly C5a levels, may serve as a clinical criterion for disease diagnosis and prediction of severity in patients with acute liver failure. Stefas et al. (13) suggest several possible functions for ApoH in viral hepatitis B pathology involved in the HBV membrane trafficking process and an organism protective function through the clearance of “oxidized” HBV.

DEGs were then used in KEGG pathway analyses and 43 pathways were screened out, such as Complement

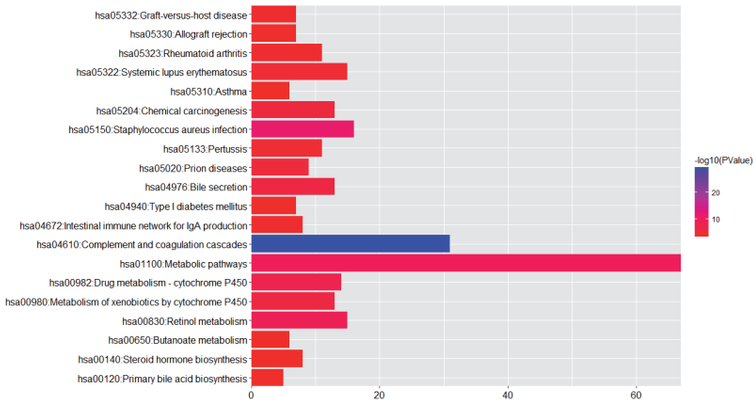

Figure 2. — The KEGG pathways and their corresponding gene number

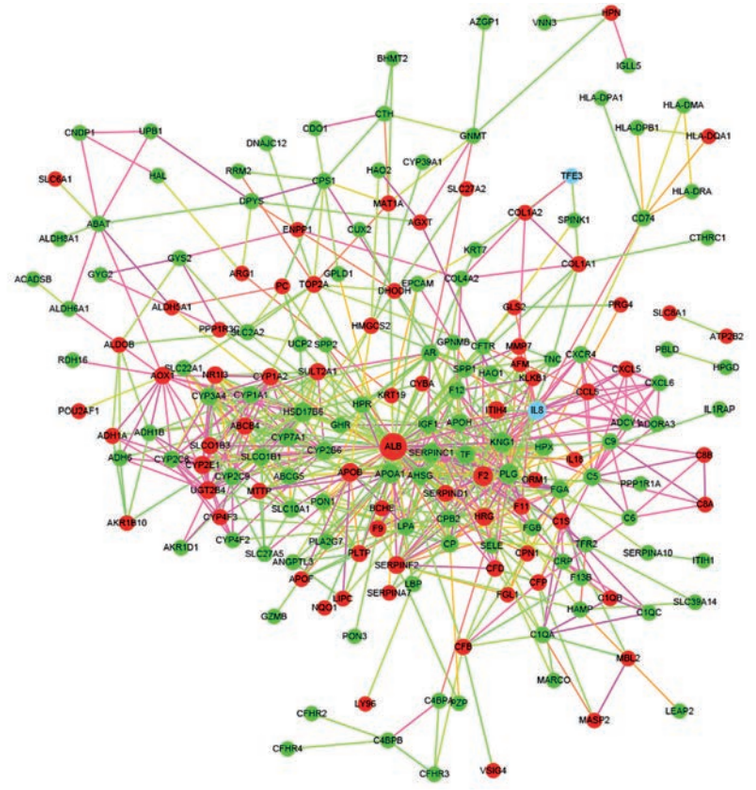

Figure 3. — The PPI network of the DEGs. The network contains 185 nodes and 692 edges. The 112 red nodes are the genes that have higher expression values in the HBV-associated acute liver failure samples compared with the control samples; the 71 bright green nodes are the genes that have lower expression values in the HBV-associated acute liver failure samples compared with the control samples; the 2 blue nodes represent the genes that have contradictory expression trend between HBV-associated acute liver failure samples and control samples.

and coagulation cascades, Intestinal immune network for IgA production, Chemical carcinogenesis. Transplanted hepatocytes produce and release tissue factor, leading to the activation of coagulation and complement pathways and eventually cell lysis. Inflammatory cells are further activated, leading to the rapid clearance of hepatocytes shortly after transplantation (14). Peroxisome proliferator-activated receptors (PPARs) are nuclear hormone receptors that are activated by fatty acids and their derivatives. PPAR has three subtypes (PPAR alpha, beta/delta, and gamma) showing different expression patterns in vertebrates. Wang et al. (15) reveal that Per1 reduces

hepatic macrophage recruitment through interaction with PPAR-γ and prevents an excessive innate immune response in endotoxin-induced liver injury.

Furthermore, the topological structure analysis of PPI network suggested that ALB, KNG1, PLG, F2 were the top 4 core genes. KNG1 (Kininogen-1) is an inhibitor of thiol proteases (16) and acts early in the intrinsic pathway of coagulation. Its function in the activation pathway includes inflammatory response and positive regulation of apoptosis. Increased levels of the proteins in the kininogen family have been often found following cell injury associated with inflammation, coagulation, fibrinolysis (17). Therefore, it was hypothesized that KNG1 may be important in the pathogenesis of HBV-associated acute liver failure via inflammatory response and positive regulation of apoptosis, which may further influence acute liver failure occurrence. The protein encoded by PLG (plasminogen) is a secreted blood zymogen that is activated by proteolysis and converted to plasmin and angiostatin. Fibrinolytic capacity was profoundly impaired in patients with acute liver failure, which was associated with decreased levels of the plasminogen and increased levels of plasminogen activator inhibitor type 1 (18). The levels of plasminogen could possibly help improve prediction with HBV-associated acute liver failure. F2 (Coagulation factor II) is proteolytically cleaved to form thrombin in the first step of the coagulation cascade which ultimately results in the stemming of blood loss. F2 also plays a role in maintaining vascular integrity during development and postnatal life. Coagulation factor II activity measured by the prothrombin time (PT) assay is used as an important prognostic marker for the severity of liver damage in paracetamol-poisoned patients (19). It is interesting to speculate that F2 may have potential as a novel prognostic marker for HBV-associated acute liver failure.

There are some limitations to our study. First, we did not generate the microarray data ourselves but took them from the GEO database. Second, validation of the results in other datasets or samples is lacking in this study, therefore, further research based on larger sample size are needed to confirm our results. This would be the next step in our research to confirm the potential function of those genes.

Conclusions

In conclusion, the results of this study may improve the understanding of the mechanism of HBV-associated acute liver failure. After analysis, a total of 328 DEGs were screened. KNG1, PLG and their relative pathway complement and coagulation cascades may be important for understanding the molecular mechanism of HBV-associated acute liver failure. However, lacking of experimental verification is a limitation of this study. In the future, these predicted results obtained from bioinformatics analysis can be verified by further experimental researches.

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