Identification of differentially expressed genes in Budd-Chiari syndrome by RNA-sequencing

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Abstract. Budd-Chiari syndrome (BCS) is an uncommon disease characterized by the occlusion or obstruction of hepatic venous outflow. The mechanism of BCS is still unclear and there are no accurate and effective diagnostic or therapeutic tools. In the present study, blood samples from BCS patients and healthy controls were used for RNA-sequencing. The differentially expressed genes (DEGs) in BCS patients compared with healthy controls were identified. Gene Ontology (GO) enrichment analysis, Kyoto Encyclopedia of Genes and Genomes pathway analysis and Protein-Protein Interaction (PPI) networks construction were performed for DEGs. A total of 405 DEGs including 317 upregulated and 88 downregulated DEGs were identified. The cytosol was the most significantly enriched GO term and the proteasome was also identified as significant enriched pathway. According to the PPI network of 30 DEGs (18 upregulated and 12 downregulated DEGs), synuclein α, tubulin β-2A class IIa and zinc finger protein Gfi-1b (GFIIB) were the three most significant hub proteins. In conclusion, several DEGs including secreted protein acidic and cysteine rich, lipocalin-2, GFI1B and proteasome-associated DEGs may be associated with the pathological process of BCS. These results can provide novel clues for the pathogenesis and provide novel diagnostic and therapeutic strategies for BCS.

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Abbreviations: APS, antiphospholipid syndrome; BCS, Budd-Chiari syndrome; DEGs, differentially expressed genes; ET, essential thrombocythemia; GFI1B, growth factor independent 1B transcriptional repressor; GO, Gene Ontology; IFI27, interferon-inducible gene 27; IVC, inferior vena cava; KEGG, Kyoto Encyclopedia of Genes and Genomes; LCN2, lipocalin-2; MF, myelofibrosis; MPDs, myeloproliferative disorders; PNH, paroxysmal nocturnal hemoglobinemia; PPI, protein-protein interaction; PV, polycythemia vera; SNCA, synuclein α; SPARC, secreted protein acidic and cysteine rich; TUBB2A, tubulin β 2A class IIa

Key words: Budd-Chiari syndrome, differentially expressed genes, RNA-sequencing, protein-protein interaction networks, myeloproliferative disorders

Introduction

Budd-Chiari syndrome (BCS) is a rare disease (1), which occurs in 1/100,000 of the general population worldwide (2). BCS is characterized by the occlusion or obstruction of the hepatic venous outflow (3), which located at the hepatic veins or on the suprahepatic portion of inferior vena cava (4). The hepatic veno-occlusive disease as well as the obstruction induced by congestive heart failure is excluded from this definition. The main symptoms of the BCS are abdominal pain, hepatomegaly and ascites (5).

BCS is a multifactorial disease and its etiology and underlying mechanism is not fully understood. However, a hypercoagulable state and thrombosis with increased blood viscosity are suspected to be the most common pathophysiological mechanism (6). Myeloproliferative disorders (MPDs) including polycythemia vera, essential thrombocythemia and paroxysmal nocturnal hemoglobinemia are prothrombotic disorders (4) that induce a hypercoagulable state (7,8), which are the major causes of BCS, and account for 40% of BCS cases (9). In addition, antiphospholipid syndrome, hyperhomocysteinemia, pregnancy, the use of oral contraceptive pills and deficiency in antithrombin III proteins C and S, may also increase the risk of BCS (10,11). Several gene mutations including the JAK2 V617F, and the factor V Leiden are also associated with BCS (12,13).

The 1-year spontaneous mortality rate of BCS is up to 70% (14). Since there is a lack of accurate and effective diagnosis and treatment in the early stages of BCS, the mortality rate still remains high, despite the current surgical approach (14). Therefore, it is crucial to develop a novel strategy for an accurate and sensitive diagnosis, and an effective therapy.

Differentially expressed genes (DEGs) in BCS patients compared with the normal controls can make a contribution to discovering the pathological progress and develop novel diagnostic biomarkers for BCS. High-throughput technologies which generate millions of reads in a short time and at a low cost were developed rapidly in recent years (15). RNA deep-sequencing (RNA-seq) can sequence cDNA and determine the RNA content in a sample by using next-generation sequencing technologies (16). RNA-seq is a powerful method to decipher global gene expression patterns and identify DEGs which have been used in many research areas (17) while this technique has not been performed to analyze and characterize the BCS transcriptome.

In the present study, RNA-seq was used to identify DEGs in the BCS and functional annotation analysis and Protein-Protein Interaction (PPI) networks construction were performed to find the BCS-associated genes and pathways. This may provide clues to pathological mechanisms, diagnostic and therapeutic strategy for BCS.

Materials and methods

Patients. Blood samples were obtained from three patients (patients 1-3) who were diagnosed with BCS, and from three healthy individuals (normal controls; NC 1-3). Patient 1 was male, 31 years old, and had been admitted to the hospital because of dizziness and fatigue for 50 days and platelet reduction for 20 days and was diagnosed with BCS (occlusion of inferior vena cava located above the hepatic vein with many collateral circulations) with splenomegaly and anemia. Patient 2 was female, 49 years old, and was hospitalized for occasional swelling of the upper abdomen, and was diagnosed with BCS (occlusion of inferior vena cava located above the hepatic vein with many collateral circulations). Patient 3 was male, 68 years old, and had been admitted to the hospital because of swelling of lower limbs and infections of lower limb ulcerations for 1 year, and was diagnosed with BCS (occlusion of inferior vena cava located near right atrium with many collateral circulations) with infections of lower limbs ulcerations, rheumatoid arthritis and chronic bronchitis. None of these three patients had prior history of medication and family history. All the participants submitted the written informed consent and the protocol was approved by the ethical committee of Shandong Jining No. 1 People's Hospital, Shandong, China.

RNA isolation and sequencing. According to the manufacturer's protocol, total RNA was isolated from blood samples using TRIzol (Invitrogen; Thermo Fisher Scientific Inc., Waltham, MA, USA). An RNeasy mini kit (Qiagen Inc., Valencia, CA, USA) was used to purify the total RNA. The quantity and integrity of purified RNA was checked using a Nanodrop ND-2000 spectrophotometer (Thermo Fisher Scientific, Inc.) and an Agilent 2100 Bioanalyzer. With a TruSeq RNA library preparation kit (Illumina Inc., San Diego, CA, USA), messenger RNA was purified from the samples (RIN>7), through oligo-d(T) probes for polyA selection. qPCR was performed using a QIAquick PCR kit (Qiagen Inc.) and the RNA-seq library was constructed. Sequencing was performed on a HiSeqTM 2500 platform (Illumina Inc.).

Identification of DEGs. By using FastQC (version 0.11.4; Babraham Institute, Cambridge, UK), the quality control of fastq data was performed (Read QC). To obtain the high quality clean data, Cutadapt version 1.9.1 (http://cutadapt.readthedocs.io/en/stable/changes.html#v1-9-1-2015-12-02) was used to remove low quality sequences, including ambiguous nucleotides and adaptor sequences. The alignment between the cleaned sequencing reads were aligned to the human genome (GRCh38.p7 assembly) by Tophat version 2.1.1 (http://ccb.jhu.edu/software/tophat/index.shtml) using the genome human University of California Santa Cruz reference annotation (www.ucsc.edu/). Cuffdiff (cole-trapnell-lab.github.io/cufflinks/cuffdiff/index.html) was used to assemble transcript and determine transcript abundance. Fragments per kilobase of exon per million fragments were mapped to determine the transcription abundancy of each gene.

Functional annotation. To further investigate the biological function of DEGs, Gene Ontology (GO) enrichment analysis, using the 'Biological Process and Molecular Function' tools, and the Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway analysis were performed by using the online software GeneCodis (genecodis.cnb.csic.es/analysis). A false discovery rate (FDR)<0.05 was defined as the criteria for statistical significance.

PPI network construction. The PPI network makes a contribution to discovering the disease-associated pathways and reforming the strategy for drug design, which is superior to the simple activation and inhibition analysis of a single protein. In the present study, the top 20 upregulated and downregulated DEGs were used to construct the PPI network by using Biological General Repository for Interaction Datasets (BioGRID) (thebiogrid.org/) and Cytoscape (www.cytoscape.org/). Proteins were represented using nodes and the interactions between two proteins were represented by edges.

Statistical analysis. Student's t-test was performed to identify the differentially expressed genes (DEGs). $P<0.05$ and abs (count_1-count_2)>100 was considered to indicate a statistically significant difference.

Results

RNA-sequencing and identification of DEGs. Blood samples from three BCS patients and three healthy controls were subjected to RNA sequencing. In total, $2.73x10^7$, $2.77x10^7$ and $2.73x10^7$ sequencing reads from BCS blood, and $2.74x10^7$, $2.68x10^7$ and $2.69x10^7$ reads from healthy blood respectively, were generated. In addition, 90.2, 89.6 and 91.3% reads from

Table I. RNA sequencing results.

Sample	Clean reads	Clean bases	Read length (bp)	Q20 (%)	GC (%)	Mapped (%)
A1	27,352,176	4,102,826,400	150	98.86;96.96	59.11	90.2
A2	27,749,880	4,162,482,000	150	98.85;97.35	58.16	89.6
A3	27,330,906	4,099,635,900	150	98.89;97.01	61.04	91.3
B1	27,420,208	4,113,031,200	150	98.71;96.98	55.60	87.7
B2	26,909,146	4,036,371,900	150	98.72;97.17	55.76	89
B3	26,754,504	4,013,175,600	150	98.82;97.02	57.73	89.8

Q20, the number of nucleotide with quality >20/nucleotide (paired-end reads: Clean read 1, read 2); GC, Guanine Cytosine number/nucleotide (paired-end reads: Clean read 1 read 2).

Table II. Top 20 upregulated and downregulated differentially expressed genes in the BCS samples.

Gene ID	Gene	NC count	BCS count	log2FC	P-value
60675	PROK2	521.102	178.57	-1.44204	$5.00x10^{5}$
387066	SNHG5	435.639	148.469	-1.62065	$5.00x10^{5}$
9172	MYOM2	472.403	32.4931	-3.86276	$5.00x10^{5}$
6231	RPS26	2,135.57	583.471	-1.90969	$5.00x10^{5}$
6235	RPS29	1,462.01	820.173	-1.19722	$5.00x10^{5}$
9381	OTOF	161.644	41.5735	-2.01754	$5.00x10^{5}$
133	ADM	363.37	174.728	-1.08298	0.0001
3429	IFI27	369.492	120.606	-1.81854	0.0001
6227	RPS21	2,030.92	1,169.11	-1.0646	0.00015
10578	GNLY	1,866.86	911.448	-1.09558	0.00015
254948	RPL9P8	629.595	300.206	-1.04615	0.0002
400499	LOC400499	674.824	290.442	-1.3079	0.00025
84525	HOPX	194.717	92.5097	-1.02509	0.0003
55007	FAM118A	305.302	147.011	-1.04756	0.00035
105378415	LOC105378415	214.944	95.7043	-1.01748	0.0004
3759	KCNJ2	333.635	163.374	-0.89589	0.00055
54541	DDIT4	510.538	284.93	-0.88093	0.00065
83999	KREMEN1	286.922	149.081	-0.94533	0.0009
60674	GAS5	259.73	133.546	-0.9557	0.00095
6170	RPL39	1,460.23	905.571	-0.86872	0.001
6622	SNCA	565.441	2,083.61	1.94496	$5.00x10^{5}$
10098	TSPAN5	137.195	527.282	2.02604	$5.00x10^{5}$
10158	PDZK1IP1	375.33	1,593.21	2.02767	$5.00x10^{5}$
6886	TAL1	88.6704	284.49	1.74036	$5.00x10^{5}$
6678	SPARC	297.068	709.309	1.31365	$5.00x10^{5}$
7280	TUBB2A	22.716	543.469	4.50119	$5.00x10^{5}$
2766	GMPR	164.324	1,066.46	2.69842	$5.00x10^{5}$
3118	HLA-DQA2	37.4776	192.71	2.3648	$5.00x10^{5}$
221895	JAZF1	127.371	274.85	1.20758	$5.00x10^{5}$
669	BPGM	101.964	238.092	1.33635	$5.00x10^{5}$
28959	TMEM176B	481.731	1,453.35	1.57701	$5.00x10^{5}$
2039	DMTN	1,493.17	5,665.35	1.86345	$5.00x10^{5}$
286	ANK1	72.7907	251.764	1.73898	$5.00x10^{5}$
759	CA1	59.5486	213.457	1.92874	$5.00x10^{5}$
54855	FAM46C	148.663	514.282	1.91142	$5.00x10^{5}$
573	BAG1	1,319.88	4,566.92	1.85414	$5.00x10^{5}$

Table II. Continued.

Gene ID	Gene	NC count	BCS count	log2FC	P-value
25853	DCAF12	628.567	2,552.27	2.10923	$5.00x10^{5}$
3934	LCN2	20.686	141.522	2.75439	$5.00x10^{5}$
8328	GFI1B	58.8981	188.993	1.65276	$5.00x10^{5}$
10133	OPTN	604.938	1,309.79	1.19751	$5.00x10^{5}$

BCS, Budd-Chiari syndrome. NC count, normal control counts in samples; BCS count, read-counts in samples of Budd-Chiari syndrome.

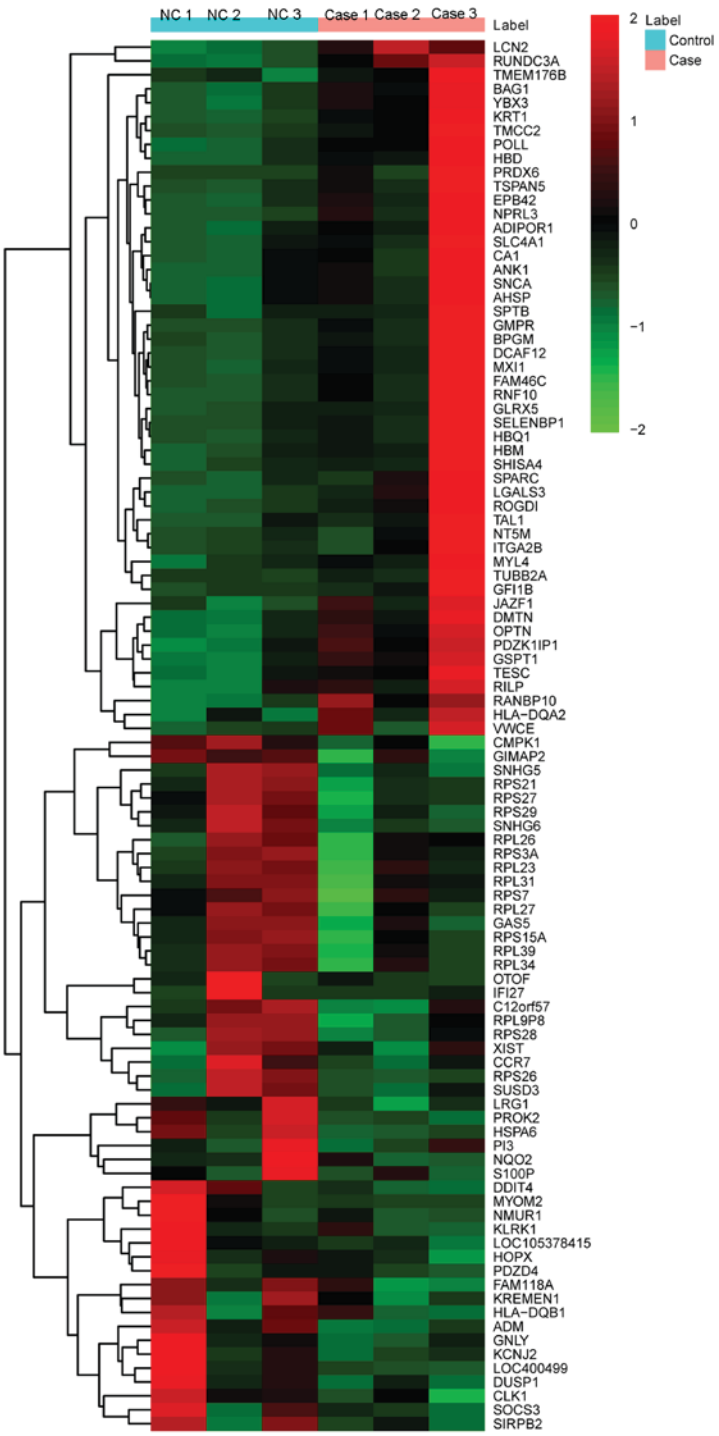

Figure 1. Heat-map of DEGs in the BCS patients compared with the healthy controls. NC, normal control; DEG, differentially expressed gene; BCS, Budd-Chiari syndrome.

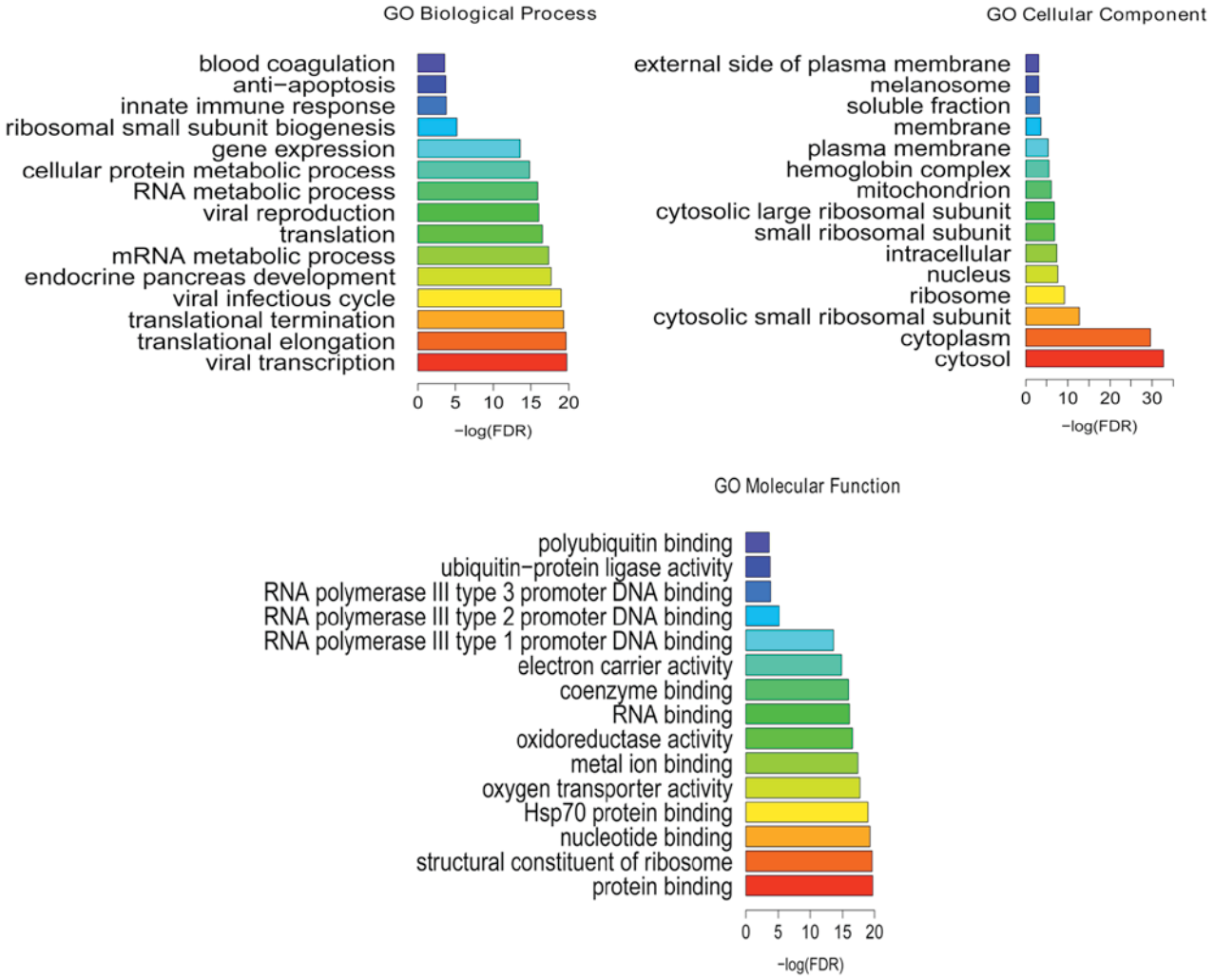

Figure 2. Top 15 most significantly enriched terms of DEGs in the BCS samples, identified by GO. DEG, differentially expressed gene; BCS, Budd-Chiari syndrome; GO, gene ontology; FDR, false detection rate.

BCS blood, and 87.7, 89 and 89.8% reads from healthy blood, respectively, were mapped (Table I).

In total, 405 DEGs were identified, including 317 upregulated and 88 downregulated DEGs with P<0.05 and abs (count_1-count_2)>100. The top 20 upregulated and downregulated DEGs are displayed in Table II. The heat-map of the top 100 DEGs is illustrated in Fig. 1.

Functional annotation. Following the GO enrichment analysis, viral transcription (FDR=1.86E-20), translational elongation (FDR=2.28E-20), protein binding (FDR=7.42E-31), structural constituent of ribosome (FDR=7.52E-16), cytosol (FDR=2.02E-33) and cytoplasm (FDR=2.38E-30) were the most significantly enriched GO terms of DEGs in the BCS samples (Fig. 2). KEGG enrichment analysis (Fig. 3 and Table III) indicated that ribosome (FDR=1.25E-17), phagosome (FDR=0.000204) and lysosome (FDR=0.000368) were the most significantly enriched pathways in the BCS samples. The proteasome (FDR=0.002124) was another significantly enriched pathway in BCS, including DEGs such as the proteasome subunit β type (PSMB) -2 and -10, proteasome subunit α type-6 (PSMA6), proteasome inhibitor PI31 subunit (PSMF1) and 26S proteasome non-ATPase regulatory subunit 2 (PSMD2).

PPI network. The PPI network of 30 DEGs (18 upregulated and 12 downregulated DEGs) were constructed including 685 nodes and 747 edges (Fig. 4). According to the PPI network, synuclein α (SNCA; degree=177), tubulin β-2A (TUBB2A; degree=89) and zinc finger protein GFI-1b (GFI1B; degree=76) were the three most significant hub proteins.

Discussion

In order to elucidate the pathogenesis of BCS at the molecular level, RNA-sequencing was performed to analyze the transcriptome of BCS patients compared with the healthy controls. A total of 405 DEGs including 317 upregulated and 88 downregulated DEGs, were identified.

Since myeloproliferative diseases (MPDs) are a leading cause for BCS (9) genes associated with MPDs may serve an important role in BCS.

A total of three MPDs-associated DEGs were identified. Secreted protein acidic and cysteine rich (SPARC) encodes a matrix-associated protein, which is involved with the maintenance and restoration of tissue homeostasis (18). Recently, SPARC was reported to serve an essential role in bone marrow stromal response to myeloproliferation. Deficiency of SPARC

Table III. Most enriched pathways identified in the BCS samples.

KEGG ID	KEGG term	No. of genes	FDR	Gene list
hsa03010	Ribosome	19	1.25E-17	RPS15A, RPS26, RPS3A, RPS25, RPL17, RPS7, RPL30, RPS21, RPL31, RPL35, RPS28, RPL26, RPL39, RPL23, RPS29, RPS27, RPL34, RPS24, RPL27
hsa04145	Phagosome	10	0.000204	HLA-DMA, NCF1, ATP6V0A1, TUBB2A, DYNC1H1, RILP, CYBB, ATP6V0C, FCGR1A, HLA-DQB1
hsa04142	Lysosome	9	0.000368	MCOLN1, GM2A, TPP1, ATP6V0A1, GUSB, DNASE2, SORT1, ATP6V0C, AP1B1
hsa04380	Osteoclast differentiation	9	0.000384	NCF1, PLCG2, RELB, LILRB1, SOCS3, CSF1R, CYBB, LILRB4, FCGR1A
hsa03050	Proteasome	5	0.002124	PSMB10, PSMA6, PSMF1, PSMB2, PSMD2
hsa00860	Porphyrin and chlorophyll metabolism	5	0.002248	FECH, GUSB, BLVRB, HMBS, ALAS2
hsa00030	Pentose phosphate pathway	4	0.00423	FBP1, GPI, G6PD, PGD
hsa04640	Hematopoietic cell lineage	6	0.005515	CD22, GP1BB, ITGA2B, CSF1R, FCGR1A, CD2
hsa00520	Amino sugar and nucleotide sugar metabolism	4	0.024957	HK1, GPI, TSTA3, RENBP
hsa05144	Malaria	4	0.024957	HBD, KLRK1, GYPC, LRP1
hsa05150	Staphylococcus aureus infection	4	0.02602	HLA-DMA, FPR2, FCGR1A, HLA-DQB1
hsa04966	Collecting duct acid secretion	3	0.031755	SLC4A1, ATP6V0A1, ATP6V0C
hsa05110	Vibrio cholerae infection	4	0.032999	PLCG2, ATP6V0A1, ATP6V0C, GNAS
hsa04910	Insulin signaling pathway	6	0.034042	HK1, FBP1, RPTOR, SOCS3, PPP1R3B, INPP5K
hsa04666	Fc gamma R-mediated phagocytosis	5	0.035841	NCF1, PLCG2, GSN, WASF2, FCGR1A
hsa00010	Glycolysis/Gluconeogenesis	4	0.043693	HK1, FBP1, GPI, BPGM

BCS, Budd-Chiari syndrome; KEGG, Kyoto Encyclopedia of Genes and Genomes; FDR, false discovery rate.

can induce myelofibrosis (MF), can suppress the activity in primary MF and enhance the myeloproliferative response to thrombopoietin (19,20). Hence, SPAR1 is a key gene which is associated with MPDs. In the present study, SPACR was upregulated in the BCS patients compared with the controls, confirming that SPACR may serve a key role in BCS as well as in MPDs. Lipocalin-2 (LCN2) is an inflammatory cytokine which is localized to myeloid cells within MF marrow cells (21). LCN2 was reported to be involved in the pathophysiological progress of MPDs (9). Compared with the controls, upregulated LCN2 was detected in the plasma of multiple MPDs patients including primary MF, PV-MF, and ET-MF patients (9). According to the present study, LCN2 was upregulated in the BCS patients as well. Another MPDs-associated DEG identified, was interferon-inducible gene 27 (IFI27). IFI27 was reported to be upregulated in the patients with MPDs (22), which was downregulated in BCS patients in the present study, but requires further investigation. Therefore, it

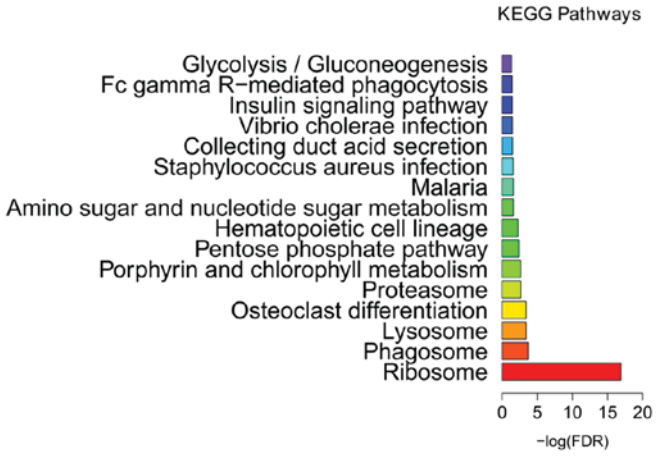

Figure 3. Most significantly enriched pathways of DEGs in the BCS samples, identified by KEGG pathway analysis. DEG, differentially expressed gene; BCS, Budd-Chiari syndrome; KEGG, Kyoto Encyclopedia of Genes and Genomes; FDR, false detection rate.

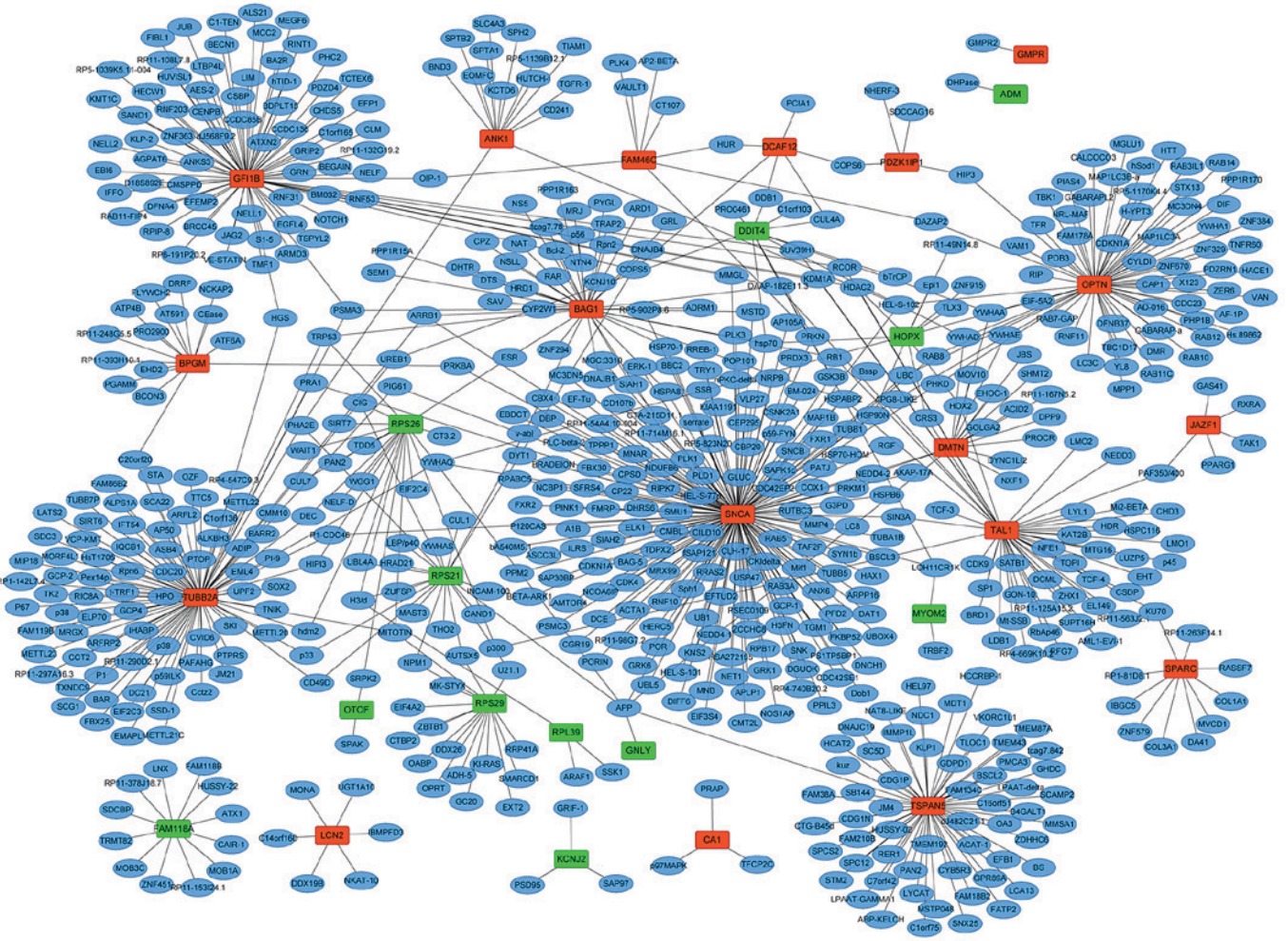

Figure 4. Protein-Protein Interaction network analysis identified 18 upregulated and 12 downregulated DEGs. Rectangles represent the proteins encoded by DEGs, the red and green colors indicate the upregulated and downregulated ones, respectively, in the BCS samples. DEG, differentially expressed gene; BCS, Budd-Chiari syndrome.

can be speculated that dysregulation of LCN2 and IFI27 may also serve an essential role in BCS affecting the progress of MPDs.

Based on the PPI network, three hub genes including *SNCA*, *TUBB2A* and *GFI1B* were identified. Among them, the *GFI1B* gene is a member of growth factor independence 1 gene family which is expressed in hematopoietic stem cells and myeloid progenitors (23). Previous studies have speculated that GFI1B may serve an important role in MPDs too (9). Because GFI1B is another DEG which identified among the top 20 upregulated DEGs it can be concluded that it may also be involved in the BCS possibly through its role function in MPDs.

According to the KEGG enrichment analysis, proteasome was a significantly enriched pathway in BCS. Proteasome was reported to support stimulated platelet function and thrombosis, and its inhibition can induce a hypothrombotic state and reduce thrombosis (10). Since the hypothrombotic state is a major mechanism of BCS, it can be concluded that proteasome may serve an essential role in BCS by regulating thrombosis, and the proteasome-associated DEGs including PSMB10, PSMA6, PSMF1, PSMB2 and PSMD2 may be closely associated with the pathological process of BCS.

In conclusion, several DEGs were identified in the BCS samples using RNA-seq, including SPARC, LCN2, IFI27 and GFI1B. Proteasome-associated DEGs may be involved in BCS through regulating the thrombosis. The results of the present study may provide a contribution to uncovering the underlying pathogenesis of BCS and to developing novel strategies for its diagnosis and treatment.

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