

# mRNA and lncRNA expression profiles of liver tissues in children with biliary atresia

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**Abstract.** Progressive liver fibrosis is the most common phenotype in biliary atresia (BA). A number of pathways contribute to the fibrosis process so comprehensive understanding the mechanisms of liver fibrosis in BA will pave the way to improve patient's outcome after operation. In this study, the differentially expressed profiles of mRNAs and long non-coding RNAs from BA and choledochal cyst (CC) liver tissues were investigated and analyzed, which may provide potential clues to clarify hepatofibrosis mechanism in BA. A total of two BA and two CC liver tissue specimens were collected, the expression level of mRNAs and lncRNAs was detected by RNA sequencing. Differentially expressed mRNAs (DEmRNAs) were functionally annotated and protein-protein interaction networks (PPI) was established to predict the biological roles and interactive relationships. Differentially expressed lncRNAs (DELncRNAs) nearby targeted DEmRNA network and DELncRNA-DEmRNA co-expression network were constructed to further explore the roles of DELncRNAs in BA pathogenesis. The expression profiles of significant DEmRNAs were validated in Gene Expression Omnibus database. A total of 2,086 DEmRNAs and 184 DELncRNAs between BA and CC liver tissues were obtained. DEmRNAs were enriched in 521 Gene Ontology terms and 71 Kyoto Encyclopedia of Genes and Genomes terms which were mainly biological processes and metabolic pathways related to immune response and inflammatory response. A total of five hub proteins (TYRO protein tyrosine kinase binding protein,

C-X-C motif chemokine ligand 8, pleckstrin, Toll-like receptor 8 and C-C motif chemokine receptor 5) were found in the PPI networks. A total of 31 DELncRNA-nearby-targeted DEmRNA pairs and 2,337 DELncRNA-DEmRNA co-expression pairs were obtained. The expression of DEmRNAs obtained from RNA sequencing were verified in GSE46960 dataset, generally. The present study identified key genes and lncRNAs participated in BA associated liver fibrosis, which may present a new avenue for understanding the patho-mechanism for hepatic fibrosis in BA.

## Introduction

Biliary atresia (BA) is a rare but severe neonatal disease characterized by an inflammatory and progressive fibrotic obstruction of the extrahepatic bile ducts resulting in cholestasis and subsequent hepatic failure if left untreated (1). Rapid liver fibrosis is a major outcome for children with BA even though they might appear normal at birth (2,3). Knowledge on the pathogenesis of liver fibrosis in BA is still limited. Considering the complicated etiologies, the identify potentially modifiable factors in BA associated liver fibrosis is an urgent need (2).

The microarray and RNA-seq technique can help to identify signatures of predominant transcriptions of the liver during fibrosis progressive in BA, which could be beneficial for uncovering new molecular mechanisms to improve the prognosis, diagnosis and treatment of this disease (4). This technique has been successfully applied for liver tissues from BA and CC to investigate the potential underlying mechanism (5-8). A transcriptional analysis of liver tissues from BA and CC identified 877 differentially expressed genes (such as COX1, SCO2, and CYTB, which are involved in oxidative phosphorylation) and several pathways with immune and inflammatory responses involved in the pathogenesis of BA (such as the Wnt-signaling pathway and TGF- $\beta$  pathway) (5) Previous studies have found that the NF- $\kappa$ B signaling pathway could regulate genes related to immune response and mediate liver fibrosis by initiating fibrosis factors and thus might serve a key role in the development of BA (9-11). Another RNA sequencing of livers from 6 BA and 6 CC found that Foxa3 may exert a role in the progression of hepatic fibrosis in BA, which may

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be a potential targeted treatment (6). Fas ligand mRNA (7), MMP7 and PCK1 (8) can be used as potential biomarkers to predict the outcome of BA and the fibrosis progression. A comparison of mRNA expression level from normal, diseased control and end-stage BA livers identified 35 genes involved in cell signaling, transcription regulation, hepatobiliary development and fibrosis process (12). The expression level of genes regulating fibrosis in liver tissues increases in infants who survived within 2 years (13). Furthermore, cDNA array demonstrate two hepato-fibrogenesis-associated genes (4), plasminogen activator inhibitor-1 (PAI-1) and tissue inhibitor of metalloproteinase-1 (TIMP-1).

Long noncoding RNAs (lncRNAs) exert specific regulatory functions in various cellular organization (14), which are involved in hepatic fibrosis by regulating gene or protein expression (15). lncRNAs can act as competing endogenous RNAs (ceRNAs) to participate in hepatic fibrosis (16). The expression level of long noncoding RNA H19 (lncRNA H19) is positively correlated with the severity of fibrotic liver injuries in BA patients, which serves a pivotal role in BA cholangiocyte proliferation and cholestatic liver injury as a sponge for let-7/HMGA2 axes and regulates S1PR2/SphK2 (17). lncRNA-Annexin A2 pseudogene 3 (ANXA2P3) is identified to participate in the process of BA-induced liver fibrosis by regulating Annexin A2 (ANXA2) (18). Our previous study on lncRNA-adducin 3 antisense RNA1 was identified as participating in liver fibrosis by targeting ADD3 *in vitro* accelerating the proliferation and migration of LX-2 cells, which are the key cells involved in hepatic fibrosis (19). The construction and analysis of regulation network based on microarray and RNA-seq results can improve understanding of the mechanisms underlying BA associated hepatic fibrosis. More evidence will pave the way to fully understand hepatic fibrosis pathogenesis in BA patients (20).

The present study aimed to identify some key DE mRNAs and DE lncRNA in liver tissues of BA patients through RNA-sequencing and also to discover some hub proteins via constructing PPI networks of DE mRNAs and DE lncRNA-DE mRNA co-expression networks. Through the aforementioned approaches, it was expected to ascertain some lncRNAs, mRNAs and pathways that serve an important role in the pathogenesis of liver fibrosis with BA.

## Materials and methods

**Patient samples.** The present study was approved by Ethics Committee on Human Research of Shenzhen Children's Hospital (approval no. 202106602) and was conducted in accordance with the principles expressed in the 1975 Declaration of Helsinki. A total of two BA infants and two age-matched choledochal cyst (CC) infants with a normal liver function were included in the present study. All the enrolled infants were confirmed diagnosed intraoperative cholangiography by the same surgical group in Shenzhen Children's Hospital. Liver biopsy tissues were collected with a written informed consent from all infants' guardians. The patient's clinical information is presented in Table I.

**RNA isolation and sequencing.** Total RNA was extracted from liver tissue samples using TRIzol® (Thermo Fisher

Table I. Clinical information in infants with BA and CC.

Variable	BA-1	BA-2	CC-1	CC-2
Age, days	72	50	1,095	857
Sex	Male	Male	Female	Female
ALP, IU/L	771	569	326	160
ALT, IU/L	37	85	52	14
AST, IU/L	196	177	57	29
TBIL, $\mu\text{mol/l}$	193.5	119.9	7.4	5.4
DBIL, $\mu\text{mol/l}$	104.2	69.8	1.2	1.1
GGT, IU/l	422	938	90	13

BA, biliary atresia; CC, choledochal cyst; ALP, Alkaline phosphatase; ALT, alanine transaminase; AST, aspartate transaminase; TBIL, total bilirubin; DBIL, direct bilirubin; GGT, gamma-glutamyl transferase.

Scientific, Inc.). Nanodrop (Thermo Fisher Scientific, Inc.) was used for preliminary qualification and quantification of RNA concentration and Agilent 2100 (Agilent Technologies, Inc.) was used for precise detection of quality of RNA library. Agarose gel electrophoresis was used to detect the integrity of retracted RNA.

After the RNA samples were qualified, rRNA was removed with Ribo-zero kit (EpiCentre; Illumina, Inc.) and then the RNA was fragmented under high temperature and metal ions. Using ribosomal-depleted RNA as a template, first-strand cDNA was synthesized with random hexamers. Subsequently, second-stranded cDNA was synthesized by adding buffer, dNTPs (dUTP, dATP, dGTP and dCTP) and enzymes, followed by purification of double-stranded cDNA using VAHTSTM DNA Clean Beads (Vazyme Biotech Co., Ltd.). The final strand-specific cDNA library was obtained through a series of experiments such as end repair, tailing, sorting, digestion of cDNA containing U by using UDG enzyme and PCR enrichment. Illumina HiSeq X Ten platform (Illumina, Inc.) sequencing was performed after pooling different libraries according to the requirements of effective concentration and target data volume. The processing and database construction for RNA-seq are in Fig. S1 and Appendix S1.

**Identification of DE mRNAs and DE lncRNAs.** The reference sequences of the corresponding species were downloaded from the database Ensemble GRCh38.p7 ([ftp://ftp.ncbi.nlm.nih.gov/genomes/Homo\\_sapiens](ftp://ftp.ncbi.nlm.nih.gov/genomes/Homo_sapiens)) for comparison with the sequencing data. Expression of mRNAs and lncRNAs was normalized and outputted with StringTie version 1.3.3b (<http://ccb.jhu.edu/software/stringtie/>).  $\log_2\text{FCI}>1$  and  $P<0.05$  were used as the cut-off criteria. Volcano plots and hierarchical clustering were used to visualize the overall distribution of differential transcripts.

**Functional annotation and pathway enrichment of DE mRNAs.** DAVID (<https://david.ncifcrf.gov>) is an online database that aggregates large numbers of genes or proteins into corresponding functional annotations and pathways, providing a quick access to various annotation data.  $P<0.05$  was used

Table II. Top 10 up- and downregulated DEmRNAs between BA liver tissues and CC liver tissues.

ID	Symbol	log2FC	P-value	Regulation
ENSG00000244734	HBB	6.99799	5.00x10 <sup>-5</sup>	Up
ENSG00000147257	GPC3	5.86627	5.00x10 <sup>-5</sup>	Up
ENSG00000159217	IGF2BP1	5.61870	5.00x10 <sup>-5</sup>	Up
ENSG00000081051	AFP	5.41660	5.00x10 <sup>-5</sup>	Up
ENSG00000068366	ACSL4	5.21616	5.00x10 <sup>-5</sup>	Up
ENSG00000276368	HIST1H2AJ	4.71279	5.00x10 <sup>-5</sup>	Up
ENSG00000169213	RAB3B	4.47424	5.00x10 <sup>-5</sup>	Up
ENSG00000274267	HIST1H3B	4.37440	5.00x10 <sup>-5</sup>	Up
ENSG00000115009	CCL20	4.33093	5.00x10 <sup>-5</sup>	Up
ENSG00000274641	HIST1H2BO	4.25590	5.00x10 <sup>-5</sup>	Up
ENSG00000140505	CYP1A2	-7.78389	5.00x10 <sup>-5</sup>	Down
ENSG00000117594	HSD11B1	-5.94395	5.00x10 <sup>-5</sup>	Down
ENSG00000122787	AKR1D1	-4.81138	5.00x10 <sup>-5</sup>	Down
ENSG00000124713	GNMT	-4.80215	5.00x10 <sup>-5</sup>	Down
ENSG00000180875	GREM2	-4.63967	5.00x10 <sup>-5</sup>	Down
ENSG00000160868	CYP3A4	-4.56139	5.00x10 <sup>-5</sup>	Down
ENSG00000205362	MT1A	-4.36469	5.00x10 <sup>-5</sup>	Down
ENSG00000134760	DSG1	-4.21303	5.00x10 <sup>-5</sup>	Down
ENSG00000171234	UGT2B7	-4.15848	5.00x10 <sup>-5</sup>	Down
ENSG00000166415	WDR72	-4.11164	5.00x10 <sup>-5</sup>	Down

DEmRNAs, differentially expressed mRNAs; FC, fold change.

as the cutoff criterion for Gene Ontology (GO) functional enrichment analysis and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway enrichment analysis.

**Protein-protein interaction (PPI) networks construction.** The STRING protein interaction database (<http://string-db.org/>) and the R language (<http://www.R-project.org/>) package STRINGdb were used for Top 100 up- and downregulated DEmRNAs protein interaction network analysis. Cytoscape software (version 3.5.0, <http://www.cytoscape.org>) was applied to visualize PPI networks.

**Nearby-targeted DEmRNAs of DElncRNAs.** The functions of lncRNAs may be related to their adjacent protein-coding mRNAs (21,22), so the protein-coding mRNAs (100-kb upstream and downstream) adjacent to lncRNAs were selected as their target mRNAs. The lncRNA-mRNA regulated networks were visualized by Cytoscape software (version 3.5.0, <http://www.cytoscape.org>).

**DElncRNA-DEmRNA co-expression networks.** DElncRNA-DEmRNA co-expression was calculated based on the dynamic changes of lncRNA and mRNA expression signal values, including expression regulation relationship and regulation direction. The DElncRNA-DEmRNA pairs with absolute values of PCC >0.99 and P<0.01 were selected and DElncRNA-DEmRNA co-expression network was constructed and visualized by Cytoscape software (version 3.5.0, <http://www.cytoscape.org>). Functional annotation and Pathway Enrichment of the DEmRNAs

co-expressed with DElncRNAs were performed with DAVID. P<0.05 was used as the criterion.

**Validation in the Gene Expression Omnibus (GEO) dataset.** The mRNA expression profiles (GSE46960) was downloaded from the GEO database (<https://www.ncbi.nlm.nih.gov/geo/>), which included a collection of 64 liver biopsy sample obtained from a patient with BA (case group) and 7 liver biopsy sample obtained from a deceased-donor adult (normal group). The expression of screened DEmRNAs obtained from our RNA sequencing were verified using the GSE46960 dataset.

**Statistical analysis.** Sequencing data were analyzed by using fold changes (FC) and Student's t-test.  $|\log_2FC|>1$  and P<0.05 were set as the criterion to identify the differentially expressed mRNAs and lncRNAs.

## Results

**DEmRNAs and DElncRNAs between BA liver tissues and CC liver tissues.** There were 2,086 DEmRNAs (1,036 up- and 1,050 downregulated) and 184 DElncRNAs (52 up- and 132 downregulated) in the BA group. The top 10 upregulated and downregulated DEmRNAs and DElncRNAs are in Tables II and III. Volcano maps of DEmRNAs and DElncRNAs are in Fig. 1A and B, respectively. Hierarchical clustering of DEmRNAs and DElncRNAs is shown in Fig. 1C and D. The raw data of the sequencing data of the present study have been uploaded to Sequence Read Archive (SRA) for SUB7923751 at <https://www.ncbi.nlm.nih.gov/sra/PRJNA701623>.

Table III. Top 10 up- and downregulated DElncRNAs between BA liver tissues and CC liver tissues.

ID	Symbol	log2FC	P-value	Regulation
ENSG00000260604	RP1-140K8.5	2.61436	5.00x10 <sup>-5</sup>	Up
ENSG00000281508	CDR1-AS	2.19782	5.00x10 <sup>-5</sup>	Up
ENSG00000270507	CTA-21C21.1	3.44985	0.00010	Up
ENSG00000232931	LINC00342	1.56982	0.00435	Up
ENSG00000259834	RP11-284N8.3	1.59598	0.00665	Up
ENSG00000225470	JPX	25.47320	0.00705	Up
ENSG00000277152	RP11-622C24.2	1.55379	0.00765	Up
ENSG00000214049	UCA1	3.00640	0.00990	Up
ENSG00000267731	RP11-147L13.8	1.79259	0.01435	Up
ENSG00000172965	MIR4435-2HG	1.87816	0.01525	Up
ENSG00000230645	AC016682.1	-4.03424	5.00x10 <sup>-5</sup>	Down
ENSG00000261578	RP11-21L23.2	-3.94999	5.00x10 <sup>-5</sup>	Down
ENSG00000250056	LINC01018	-2.43565	0.00070	Down
ENSG00000248709	CTC-505O3.2	-3.32413	0.00100	Down
ENSG00000236378	RP11-394G3.2	-8.03214	0.00115	Down
ENSG00000234509	AP000253.1	-3.26890	0.00145	Down
ENSG00000251637	RP11-119D9.1	-3.81996	0.00275	Down
ENSG00000261012	RP11-116D2.1	-1.07168	0.00285	Down
ENSG00000260855	RP11-439E19.10	-1.57691	0.00395	Down
ENSG00000234456	MAGI2-AS3	-2.02662	0.00420	Down

DElncRNAs, differentially expressed lncRNAs; FC, fold change.

**Functional annotation of DEmRNAs.** To improve understanding of the biological functions of DEmRNAs, GO and KEGG enrichment analyses were performed. Oxidation-reduction process ( $P=1.52 \times 10^{-21}$ ), immune response ( $P=3.10 \times 10^{-17}$ ), inflammatory response ( $P=4.12 \times 10^{-16}$ ) and metabolic process ( $P=1.09 \times 10^{-11}$ ) were significantly enriched GO biological process in BA (Fig. 2A). Extracellular exosome ( $P=3.47 \times 10^{-45}$ ) and mitochondrial matrix ( $P=1.35 \times 10^{-18}$ ) were significantly enriched GO cellular component in BA (Fig. 2B). Electron carrier activity ( $P=5.30 \times 10^{-16}$ ) and pyridoxal phosphate binding ( $P=1.14 \times 10^{-12}$ ) were significantly enriched GO molecular function in BA (Fig. 2C). Metabolic pathways ( $P=4.63 \times 10^{-18}$ ), Biosynthesis of antibiotics ( $P=5.88 \times 10^{-17}$ ), Glycine, serine and threonine metabolism ( $P=2.19 \times 10^{-13}$ ) and complement and coagulation cascades ( $P=3.19 \times 10^{-12}$ ) were top 4 enriched KEGG pathways in BA (Fig. 2D).

**Protein-protein interaction (PPI) networks.** The PPI networks contained 176 nodes and 665 edges. TYRO protein tyrosine kinase binding protein (degree=29), C-X-C motif chemokine ligand 8 (degree=27), Toll-like receptor (TLR) 8 (degree=24), pleckstrin (degree=23), C-C motif chemokine receptor (CCR)5 (degree=22), CCR1 (degree=22), lysosomal-associated protein transmembrane 5 (degree=21), TLR7 (degree=21), cytochrome P450 family 3 subfamily A member 4 (degree=21) and CD53 molecule (degree=20) were the top 10 hub proteins of the PPI networks (Fig. 3).

**DElncRNAs-nearby-targeted DEmRNAs.** There were 31 DElncRNAs-nearby-targeted DEmRNA pairs in total, of

which 31 DElncRNAs and 30 DEmRNA were detected (Fig. 4). CTD-2256P15.2 and XLOC\_045318 all nearby CMBL.

**DElncRNA-DEmRNA co-expression networks.** There were 2,337 DElncRNA-DEmRNA co-expression pairs in total, of which 54 DElncRNAs and 848 DEmRNAs were identified with an absolute value of PCC >0.99 and  $P < 0.01$  (Fig. 5). The positive regulated network is shown in Fig. 5A and the negative regulated network shown in Fig. 5B and C.

**Functional annotation of DEmRNAs co-expressed with DElncRNAs.** Inflammatory response ( $P=1.04 \times 10^{-12}$ ), immune response ( $P=1.64 \times 10^{-09}$ ), neutrophil chemotaxis ( $P=3.03 \times 10^{-08}$ ) and oxidation-reduction process ( $P=1.85 \times 10^{-07}$ ) were significantly enriched GO terms (Fig. 6A). Extracellular exosome ( $P=2.14 \times 10^{-24}$ ) and plasma membrane ( $P=2.16 \times 10^{-10}$ ) were significantly enriched GO cellular component in BA (Fig. 6B). Oxidoreductase activity ( $P=3.77 \times 10^{-05}$ ) and aldehyde dehydrogenase activity ( $P=6.74 \times 10^{-05}$ ) were significantly enriched GO molecular function in BA (Fig. 6C). Complement and coagulation cascades ( $P=2.57 \times 10^{-07}$ ), Fatty acid degradation ( $P=2.85 \times 10^{-06}$ ), Drug metabolism-cytochrome P450 ( $P=1.20 \times 10^{-04}$ ) and Metabolic pathways ( $P=2.68 \times 10^{-04}$ ) were top 4 significantly enriched KEGG pathways (Fig. 6D).

**Validation of selected DEmRNAs in GEO database.** An mRNA expression profile was downloaded from GEO database and the expression patterns of DEmRNAs obtained from our RNA sequencing were verified. As shown in Fig. 7A and B, 111 mRNAs in upregulated DEmRNAs and

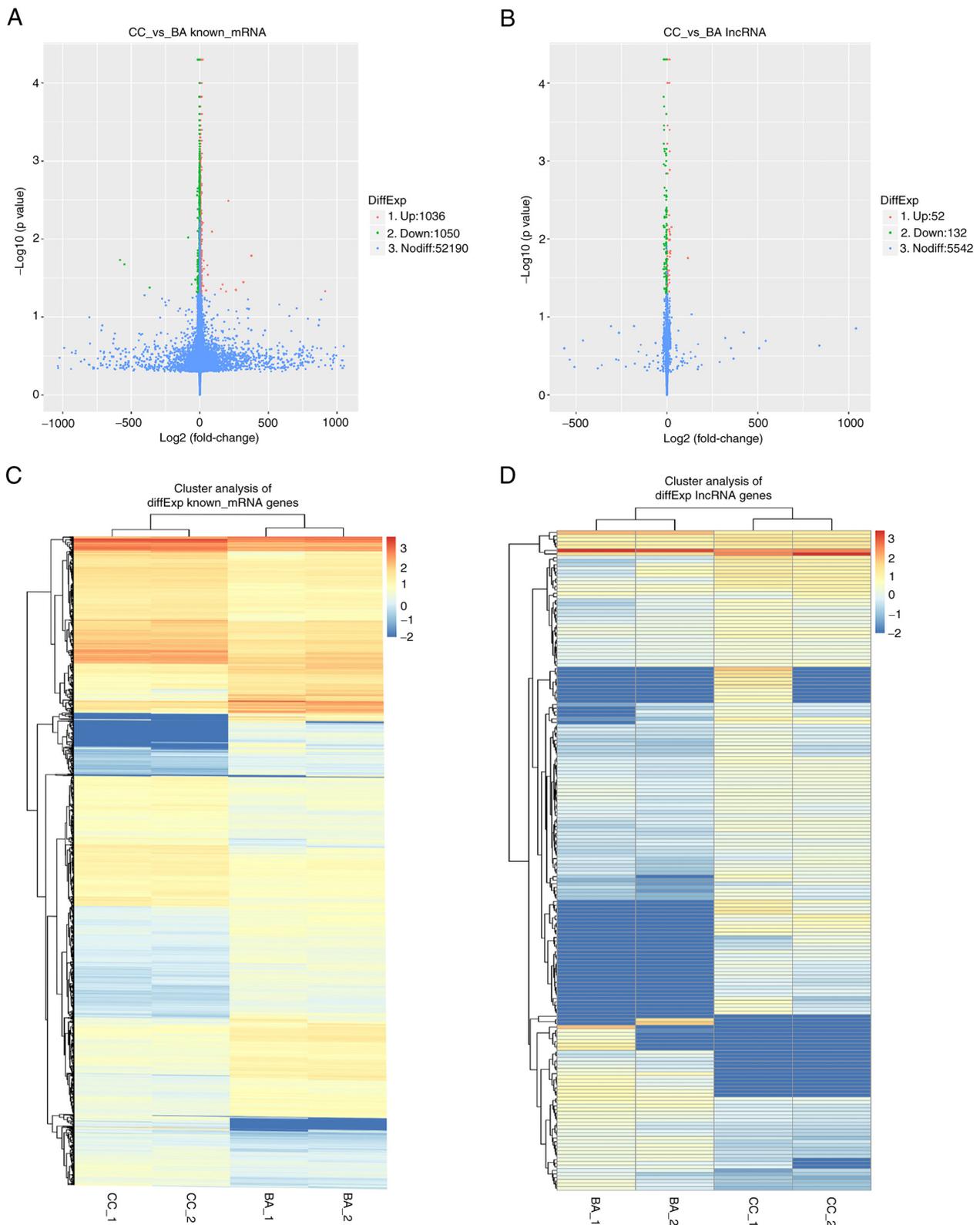


Figure 1. DE mRNAs and DE lncRNAs between BA liver tissues and CC liver tissues. Volcano spot results of (A) DE mRNAs and (B) DE lncRNAs between BA liver tissues and CC liver tissues. Red spot and green spot respectively indicated up- and downregulated genes, blue spot indicated no significant difference. Hierarchical clustering results of (C) DE mRNAs and (D) DE lncRNAs between BA liver tissues and CC liver tissues. DE mRNAs/DE lncRNAs and tissue samples were displayed as row and column, respectively. The color scale represented the expression levels. DE mRNAs, differentially expressed mRNAs; DE lncRNAs, differentially expressed lncRNAs; BA, biliary atresia; CC, choledochal cyst.

102 mRNAs in downregulated DE mRNAs were verified. Then when the present study focused on the top 10 upregulated DE mRNAs in BA from the results (Table II), five of them

(HBB, GPC3, IGF2BP1, AFP and CCL20) were consistent with the GEO database in terms of expression patterns of mRNA (Fig. 7C). Similarly, mRNAs of CYP1A2, HSD11B1,

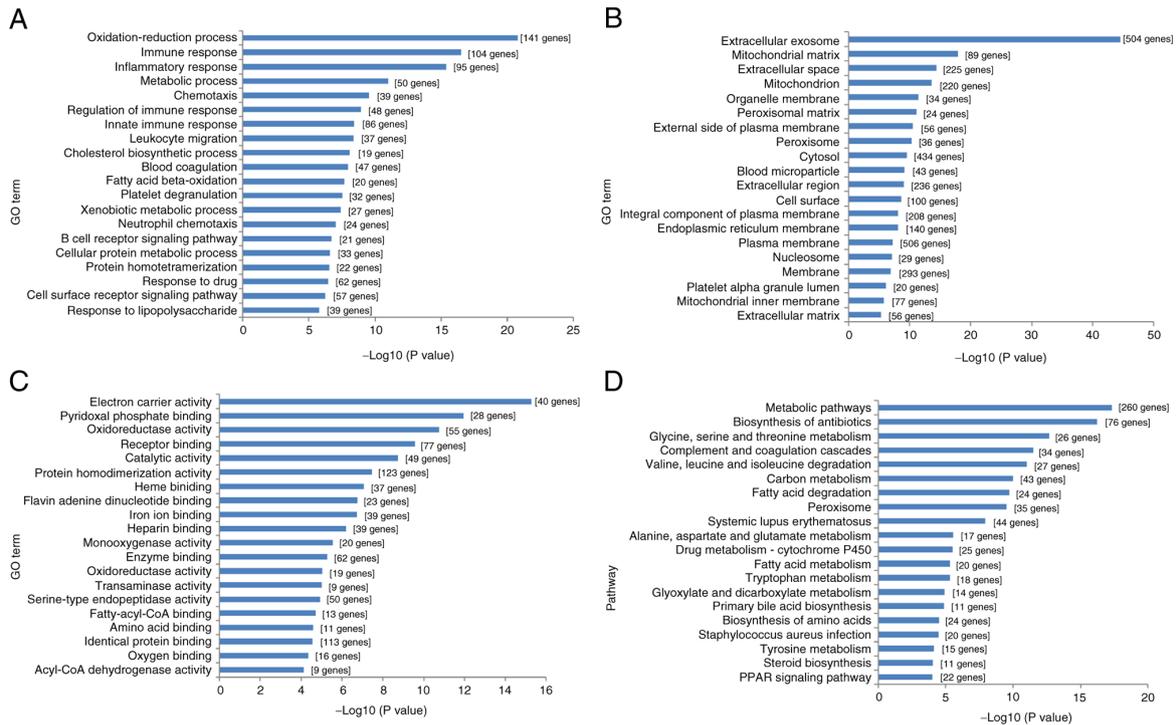


Figure 2. Top 20 significantly enriched GO terms and top 20 KEGG pathways of DEMRNAs between BA liver tissues and CC liver tissues. The x-axis is p-value for each GO term or pathway as  $-\log_{10}$  and the y-axis indicates (A) biological process GO term, (B) cellular component GO term, (C) molecular function GO term or (D) KEGG pathway. GO, Gene Ontology; KEGG, Kyoto Encyclopedia of Genes and Genomes; DEMRNAs, differentially expressed mRNAs; BA, biliary atresia; CC, choledochal cyst.

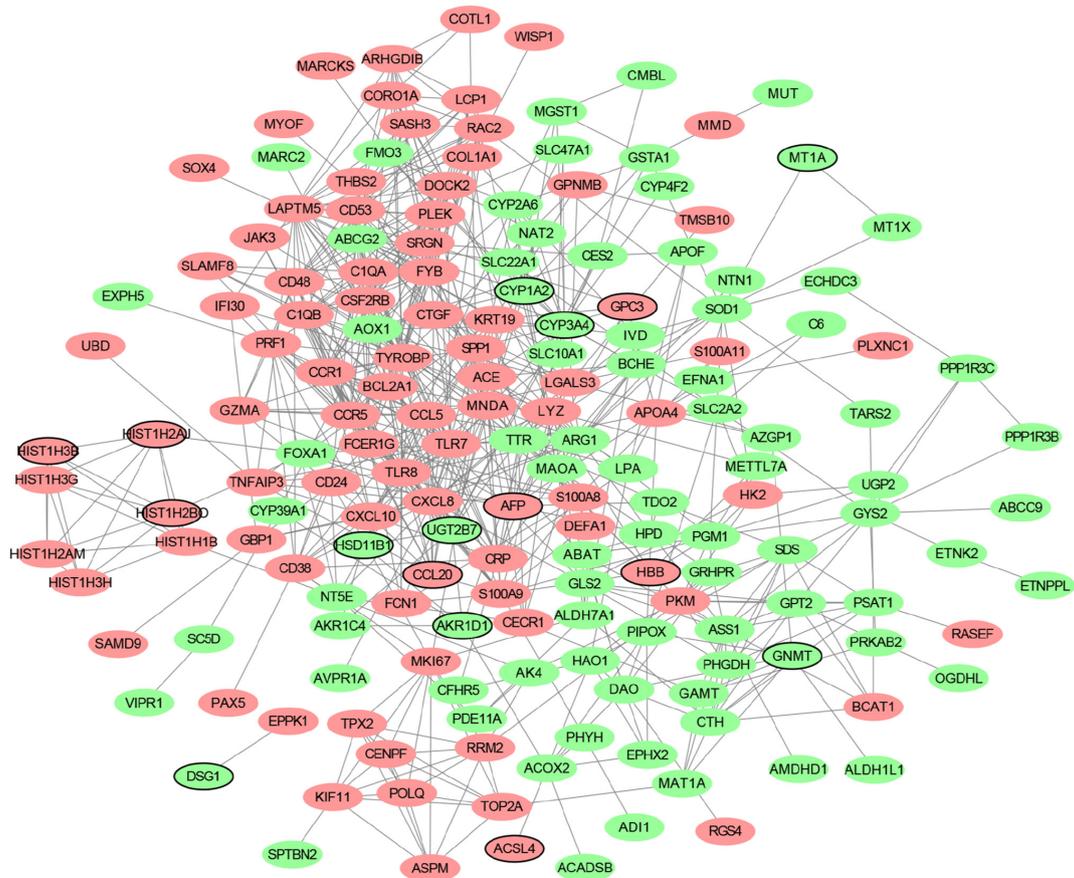


Figure 3. PPI networks. Proteins encoded by up- and downregulated DEMRNAs between BA liver tissues and CC liver tissues were displayed as red and green ellipses. DEMRNAs derived from top 10 up- and down-regulated DEMRNAs between BA liver tissues and CC liver tissues were displayed in ellipses with a black border. PPI, protein-protein interaction; DEMRNAs, differentially expressed mRNAs; BA, biliary atresia; CC, choledochal cyst.



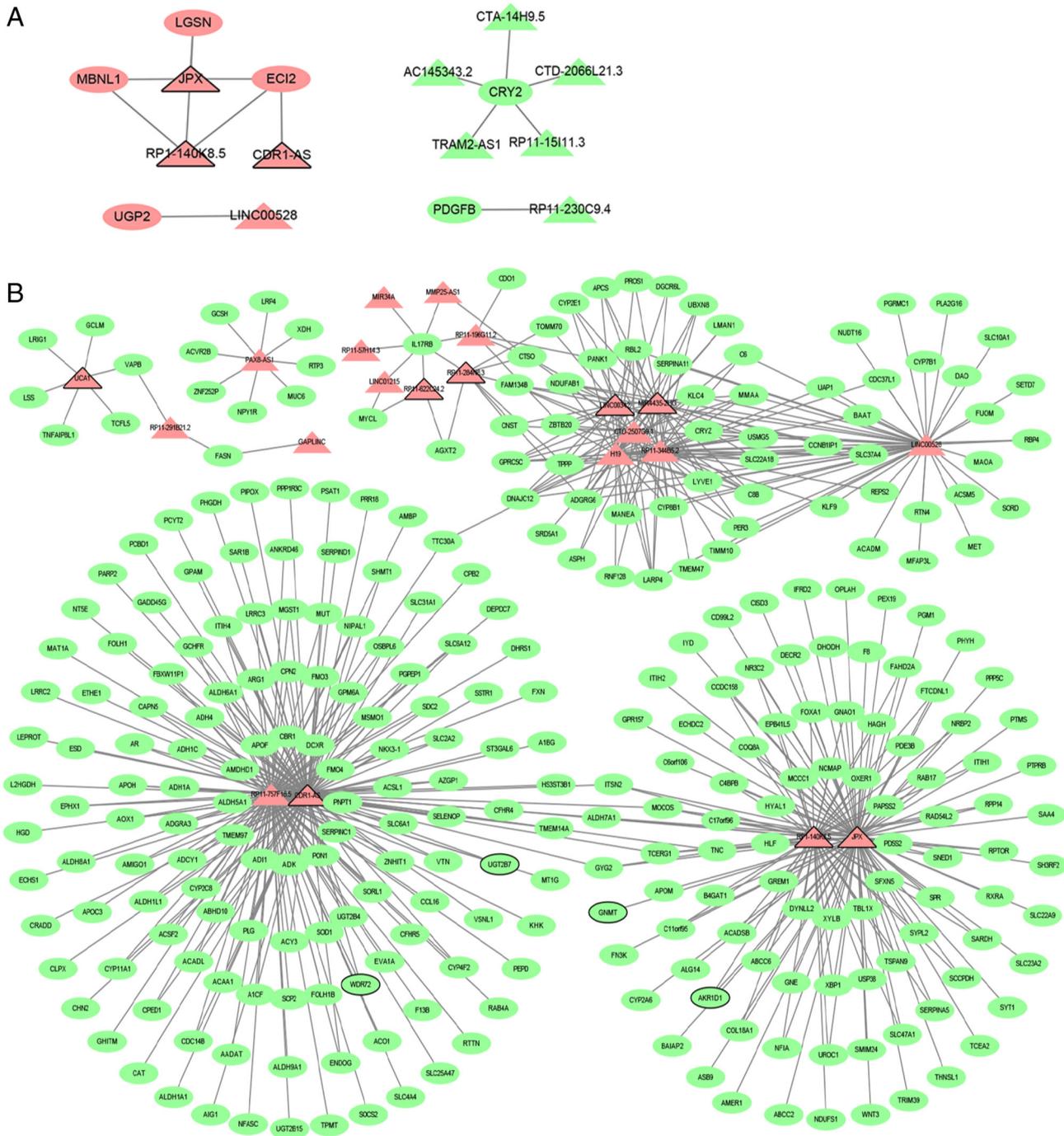


Figure 5. Continued.

Wnt/ $\beta$ -catenin signaling. Sirisomboonlarp *et al.* (29) found that circulating GPC3 protein is significantly elevated in BA patients and positively correlated with liver stiffness, indicating that serum GPC3 could be a biomarker to evaluate hepatic function and to predict prognosis for BA patients following Kasai surgery. Notably, from the results of the present study, GPC3 was also identified to be the second most over-expressed DEMRNAs in BA liver tissues.

Serum  $\alpha$ -fetoprotein (AFP) was strongly associated with epithelial liver tumors (30). It has a sensitivity of >90 and >70%, for hepatocellular carcinoma and hepatoblastoma (HB), respectively (31). Amir *et al.* (32) found that patients with BA

are far more prone to suffer from HB and in such cases, serum AFP levels might not be specific to diagnose HB in such cases, though remaining sensitive. A previous study (33) identified that ~8% of BA children were affected by liver tumors and AFP level screening and regular imaging examinations were crucial for early diagnosis of malignant liver tumors in BA patients.

Distinguishing BA from other non-BA neonatal cholestasis is challenging (34). Shteyer *et al.* (35) show that Continuous Breath  $^{13}\text{C}$ -methacetin breath test might be useful to distinguish BA from non-BA. Methacetin is a substrate that can evaluate liver metabolic function by being metabolized by cytochrome P450 1A2 (CYP1A2) (36), which is negatively

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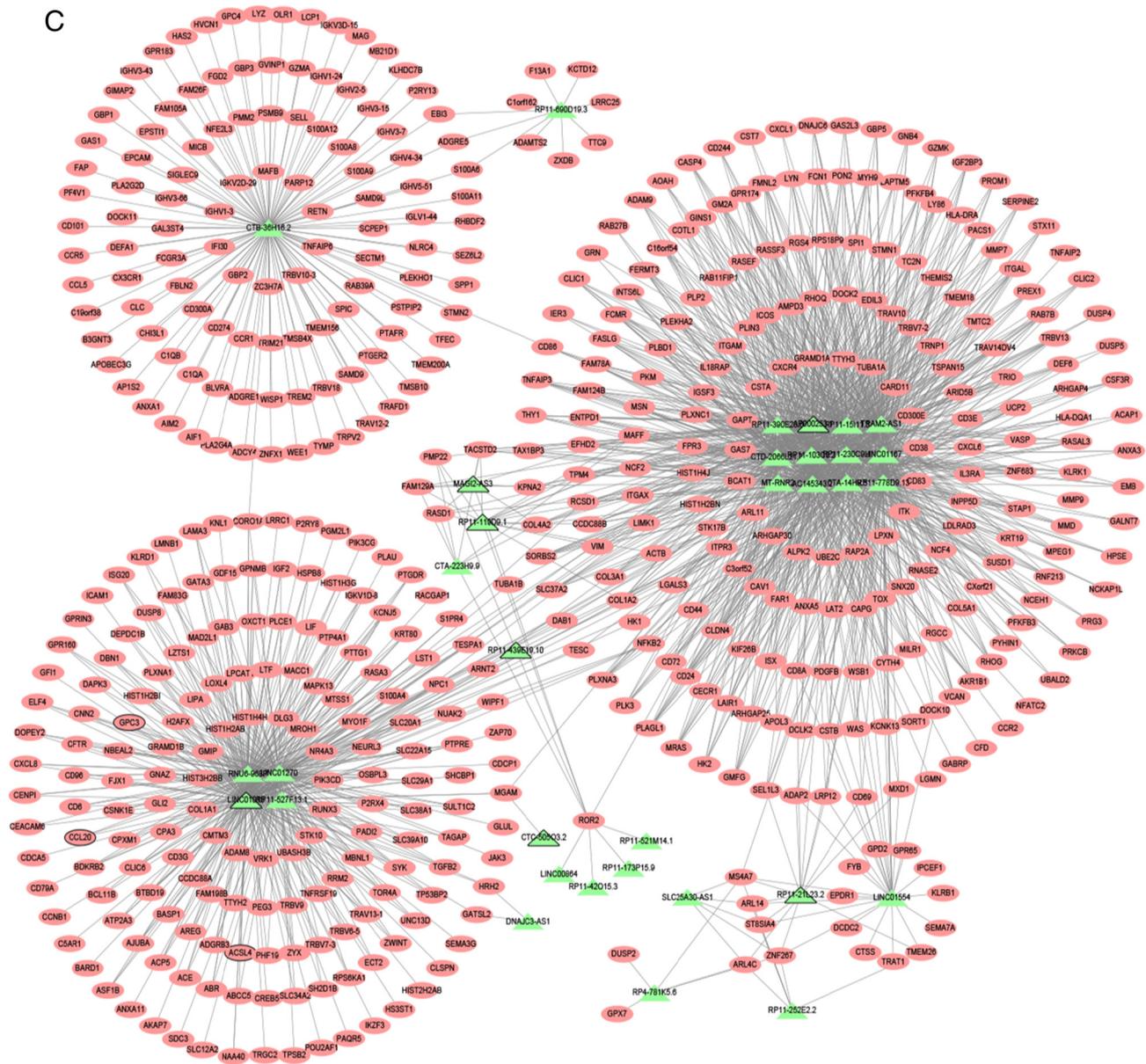


Figure 5. DElncRNA-DEmRNA co-expression networks, including (A) positive regulatory and negative regulated networks. (B and C). DElncRNAs and their nearby DEmRNAs between BA liver tissues and CC liver tissues were shown in triangles and ellipses, respectively. Up- and downregulation in BA liver tissues compared with CC liver tissues were displayed in red and green color, respectively. DElncRNAs/DEmRNAs derived from top 10 up- and downregulated DElncRNAs/DEmRNAs were displayed in triangles and ellipses with a black border. DElncRNAs, differentially expressed lncRNAs; DEmRNAs, differentially expressed mRNAs; BA, biliary atresia; CC, choledochal cyst.

correlated with hepatic fibrosis in patients with chronic hepatitis C viral infection (37,38) and is also found to be involved in other forms of hepatic diseases (39). Crawford *et al* (40) discovered that downregulated *CYP1A2* could aggravate inflammatory response due to an increase of proinflammatory cytokines, such as  $TNF-\alpha$  and *IL-6*. *CYP1A2* is the most significant downregulated DEmRNAs in BA liver tissues, indicating that it might participate in this inflammatory fibrosclerosing disease and thus can be an indicator to differentiate BA. Therefore, further investigations into the intrinsic pathway of *CYP1A2* on the etiology of progressive fibrosis in BA are of importance.

It is hypothesized that the discovery of more valuable biomarkers to monitor the progression of hepatic fibrosis

following KPE may help improve clinical outcomes in patients and delay the need for liver transplantation (41), including lncRNA *APTR* (42), miR-21 (43) and mRNA *FN14* (44). In the present study, the results also identified a number of valuable biomarkers related to liver fibrosis. Chemokine ligand-20 (*CCL20*) acted as a potent chemoattractant for immature dendritic cells, which can mediate the strong inflammatory responses that drive liver fibrosis (45). *In vivo* experiments show that a deficiency of 11 $\beta$ -hydroxysteroid dehydrogenase-1 (11 $\beta$ HSD1) could lead to the activation of hepatic myofibroblast and thus exacerbate liver fibrosis in mice (46). Expression level of steroid 5 $\beta$ -reductase (*AKR1D1*) in the liver tissue is negatively correlated with liver fibrosis and inflammation (47). Glycine N-methyltransferase (*GNMT*) is the most abundant

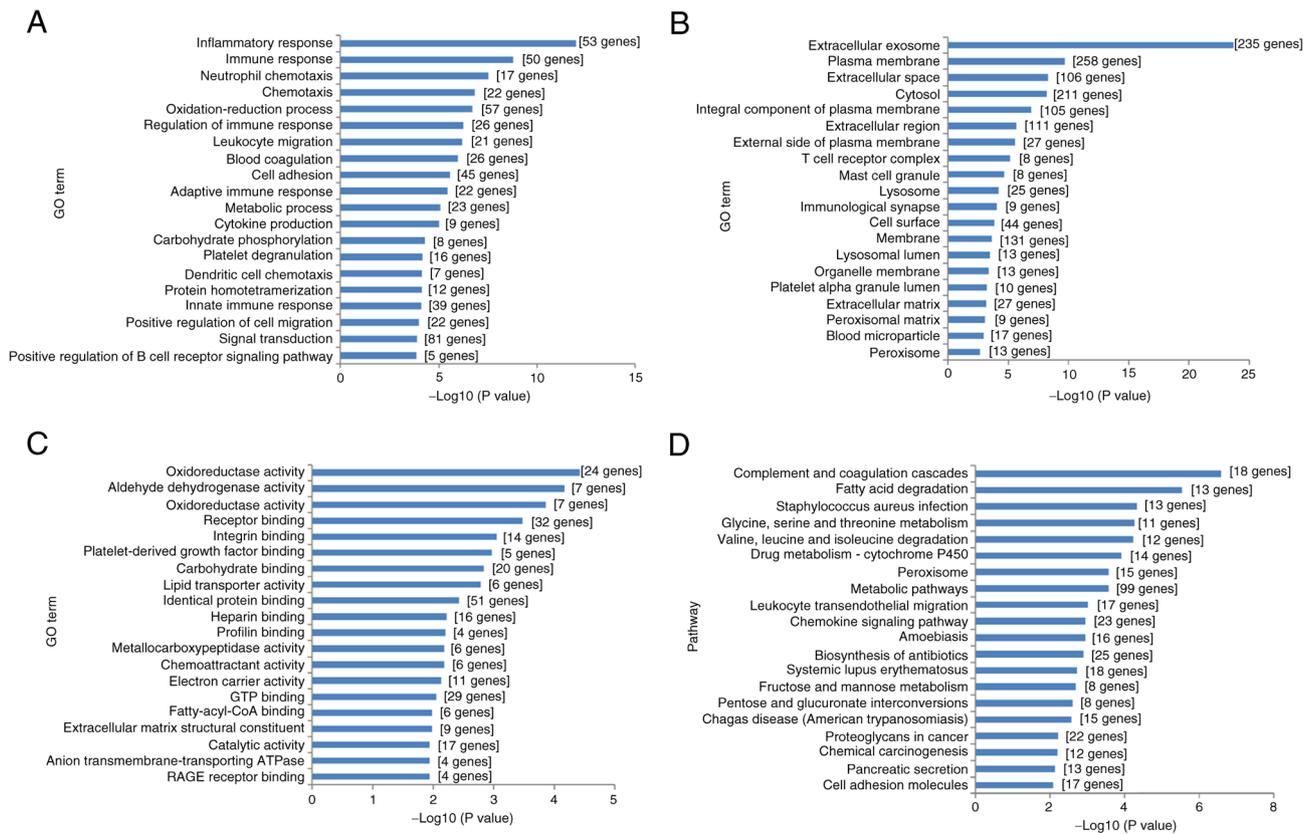


Figure 6. Top 20 significantly enriched GO terms and KEGG pathways of DEmRNAs co-expressed with DELncRNAs between BA liver tissues and CC liver tissues. The x-axis is P-value for each GO term or pathway as  $-\log_{10}$  and the y-axis indicates (A) biological process GO term, (B) cellular component GO term, (C) molecular function GO term or (D) KEGG pathway. GO, Gene Ontology; KEGG, Kyoto Encyclopedia of Genes and Genomes; DEmRNAs, differentially expressed mRNAs; BA, biliary atresia; CC, choledochal cyst.

methyltransferase in the liver and its reduction has a negative effect on the maintenance of normal liver functions progressing to fibrosis, cirrhosis and hepatocellular carcinoma (48).

Liver fibrosis is strongly associated with the activation of hepatic stellate cells. LncRNA XIST enhances eol-induced autophagy process and subsequently activates hepatic stellate cells through the miR-29b/HMGB1 signaling pathway (49). LncRNA JPX, a non-protein coding RNA transcribed by the X deactivated central gene, activates XIST transcription via an interaction with CCCTC-binding factor (50). From the sequencing data, the present study identified that JPX was an upregulated DELncRNA. Although the regulatory relationship between JPX and XIST was established, the role it serves in hepatic fibrosis and BA remains to be explored in the future.

Kerola *et al* (51) found a decrease of the expression level of TGF- $\beta$ 1 after successful HPE, indicating that TGF- $\beta$ 1 might be involved in exacerbating liver fibrosis. LncRNA miR4435-2HG can promote prostate carcinoma (52), non-small-cell lung carcinoma (53) and ovarian carcinoma (54) by interacting with TGF- $\beta$  signaling and thus LncRNA miR4435-2HG-mediated TGF- $\beta$ 1 signaling pathway might be involved in liver fibrosis of BA.

Second, Cytoscape was used to select the top 10 hub genes of PPI network for further analysis and it revealed that CXCL8, TLR8, CCR5, TLR7 and CYP3A4 were associated with BA, revealing that these hub genes may be involved in the occurrence of BA.

CXCL8 regulates inflammation and immune response via chemoattractance to neutrophils (55). Studies show that an elevation of serum CXCL8 together with its increase in liver may participate in inflammation and liver fibrosis in patients with BA and thus CXCL8 may be a potential biomarker for diagnosis, severity evaluation and outcome of BA (56,57).

The major inappropriate host immunological reactions against unknown ligands via the TLR cascades may trigger progressive inflammatory biliary destruction that manifests as BA in newborns or infants. TLR cascades may induce an excessive immune reaction against multiple ligands in the bile duct system, leading to a progressive destruction and inflammatory fibro-obstruction of biliary ducts seen in BA. miR expression level of TLR8 is significantly elevated in liver tissues in the early BA group (58). The activation of TLR7 signaling pathway is crucial for cholangiocytes proliferation in rhesus rotavirus (RRV)-infected mice models (59). In addition, TLR7 can recognize infectious pathogens, activated type 1 interferon and induce the expression of inflammatory response genes, including IL-8 (60).

A previous study has shown that CC chemokines, including CCL2, CCL3, CCL5, stimulate THP-1 cells to increase MMP-9 protein levels in a dose-dependent manner, indicating that the activation of immune cells and MMP are closely related to the occurrence of tissue inflammation (61). Leonhardt *et al* (62) identified >40 significantly differentially-expressed genes in BA mice. Most of the upregulated genes in BA-positive mice

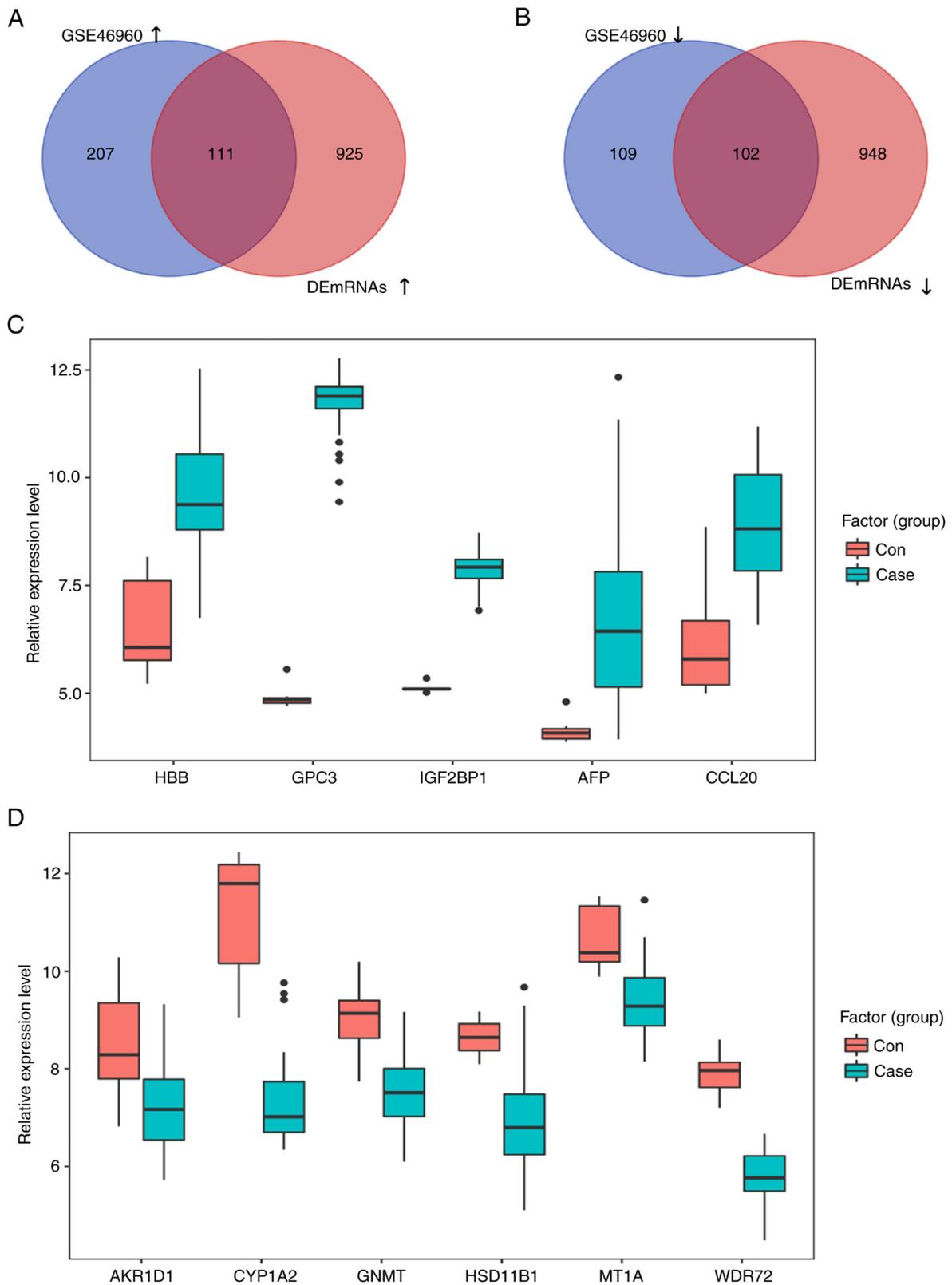


Figure 7. Validation of selected DEmRNAs in GEO database. Venn diagrams indicating that (A) 111 upregulated and (B) 102 downregulated DEmRNAs were consistently in both expression profiles. Boxplot showed (C) 5 upregulated DEmRNAs and (D) 6 downregulated DEmRNAs expression patterns; GEO, Gene Expression Omnibus.

were involved in immune response, such as *CCL2*, *CCL5*, *CCR5*, *CXCL9*, *CXCL10* and *IL1F5*. To clarify the pathogenesis of BA, the immune reaction during the process of liver fibrosis should be further examined.

Finally, lncRNA-targets were analyzed. In order to fully illustrate how lncRNAs participate in a disease, it is widely accepted to perform a lncRNA-mRNA co-expression analysis to explore the biological functions of lncRNAs by evaluating

and analyzing their co-expressed mRNAs. In the co-expression networks of the present study, CYP2E1 was a target for H19, H19 was upregulated and CYP2E1 was downregulated. Xiao *et al.* (17) discovered that the levels of H19 in both liver and serum exosome increase in line with the severity of fibrosis in patients with BA. De Bock *et al.* (63) found that CYP1A2, 2C19, 2E1 and 3A4 were at low activity levels in BA patients, which is consistent with the results of the present study. As for how they regulate each other in hepatic fibrosis and BA, this remains to be explored in the future.

A total of 2,086 DE mRNAs and 184 DE lncRNAs were identified between liver tissues in BA and CC. DE mRNA and DE lncRNA including GPC3, AFP, CYP1A2, CYP3A4, JPX and miR4435-2HG, might have an important role in the pathogenesis of liver fibrosis in BA. Furthermore, the hub genes of PPI network and the relation pairs in the lncRNA-target network were analyzed, including hub gene CXCL8, TLR7, TLR8, CCR5 and lncRNA-target pair H19 CYP2E1.

The mechanisms of fibrogenesis in BA remain to be elucidated, multiple pathways are simultaneously activated under cholestasis background. It is hoped that the RNA-sequencing results and bioinformatics analysis in the present study could contribute some indications for description of this pathological process.

There are some limitations to the present study; first, as in any statistical analysis, a reliable result depends on large-scale samples. The sample size in the present study was relatively small, due to the rare incidence of this progressive and severe neonatal fibro-obstructive cholangiopathy. Second, two age-unmatched patients with choledochal cyst under normal liver function test was as control instead of normal liver tissues due to ethical issues. It is hoped that further studies can be performed to validate the findings of the present study by enrolling more clinical samples and then the possible mechanisms can be clarified more accurately. Third, experiments on these candidate hub genes and pathways of the potential relation pair and network crosstalking to elucidate the molecular mechanism of BA are required.

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### Availability of data and materials

The data generated and analyzed in the present study may be found in the Sequence Read Archive (SRA) under accession number (SUB7923751) at the following URL: <https://www.ncbi.nlm.nih.gov/sra/PRJNA701623> and the GSE46960 dataset obtained from GEO at <https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE46960>. Other data

used and/or analyzed during the current study are available from the corresponding author on reasonable request.

### Authors' contributions

YY and BW confirm the authenticity of all the raw data. TL and BW conceived and designed the present study. YY collected liver tissues and clinical data. YY and WeiW performed the experiments. WenW and WeiW analyzed the data. YY and WenW interpreted the data. WenW and WeiW drafted the manuscript. WenW and TL prepared Fig. 1-7 and Tables II and III. YY and WeiW prepared Table I. YY, TL and BW reviewed and revised the manuscript. All authors read and approved the final manuscript.

### Ethics approval and consent to participate

The study was approved by Ethics Committee on Human Research of the Faculty of the Shenzhen Children's Hospital (approval no. 202106602). All tissue samples were collected with written informed consent from all participants or parents/guardians in the case of children under 18 and was conducted based on the principles expressed in the 1975 Declaration of Helsinki.

### Patient consent for publication

Not applicable.

### Competing interests

The authors declare that they have no competing interests.

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