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Original Article

Gene expression profiling of extrahepatic ducts in children with biliary atresia

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Abstract: As an inflammatory obliterative cholangiopathy of neonates, biliary atresia (BA) affects both intrahepatic and extrahepatic bile ducts. Its etiology has remained largely unknown. Gene expression profiling was conduced for extrahepatic bile duct tissues (including porta hepatis & common bile duct) to identify novel targets for further studies of BA. Among these tissues, porta hepatis was regarded as fibrosis group while common bile duct as self-control group. The analysis of gene expression profile in these tissues was performed with Affymetrix human microarray. Quantitative RT-PCR (qRT-PCR) was performed to confirm these results. The differential expressions of genes were identified through fold-change filtering. Gene Ontology (GO) and pathway analyses were performed using standard enrichment computation method. It was found that a total of 140 genes were differentially expressed between porta hepatis and common bile duct tissues, 19 genes up-regulated and 121 genes down-regulated. Moreover, GO analysis found that cell adhesion molecules, extracellular matrix formation, protein digestion & absorption functions may be involved in the pathogenesis of porta hepatis fibrosis. Lastly the qRT-PCR data confirmed that IL7 and CLDN2 were significantly up-regulated and both might be involved in the etiology of BA, the expression level of VCAM1 was positively correlated with severity of liver fibrosis in the BA infants. Our results demonstrated that the expressions of these aberrant genes responding to fibrosis in porta hepatis of patients with BA. Further studies of these genes may provide useful insights into the pathological mechanisms of BA.

Keywords: Biliary atresia, gene expression profiling, microarray, porta hepatis fibrosis

Introduction

Biliary atresia (BA), a progressive, sclerosing, inflammatory process in children, leads to cirrhosis and death if untreated [1-3]. As a devastating disease of intrahepatic and extrahepatic bile ducts, it is characterized by periductular inflammation and fibrosis and associated with progressive obliteration of bile ducts during the first few weeks of life [4-6]. Although the cause and pathogenesis of BA have remained largely unknown, genetic induction of proinflammatory immunity was assumed to play a pivotal role [7, 8]. Development of biliary system is a unique process thoroughly reviewed in several recent papers [9, 10]. The ventral foregut endoderm develops two protrusions: cranial part leads to the formation of intrahepatic bile ducts while caudal part generates extrahepatic biliary tree, including hilar bile duct, cystic duct and common bile duct [11]. The process of BA leads to fibrosis in hilar bile duct. However, cystic duct and common bile duct are always spared in BA infants.

Genetic factors may contribute to the development of BA [12, 13]. BA may result from an inherent defect in the epithelial-mesenchymal signaling pathways so that there is an improper formation of bile ducts at porta hepatis during the first trimester [14]. Jorge A Bezerra employed gene microarrays for identifying differentially expressed genes in liver specimens of infants with BA. There was a coordinated activation of genes involved in lymphocyte differentiation [7]. Some other studies examined the gene expression profiling of livers from BA patients [15-18]. However, none of the above reports was designed to identify genes playing a key role in the pathogenesis of fibrosis in porta hepatis with BA.

For the question of whether alterations in differentially expressed genes play a role, gene

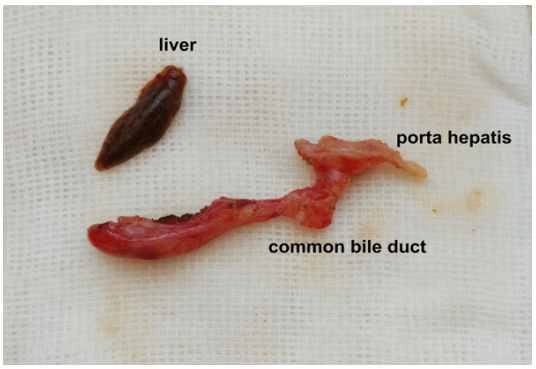

Figure 1. Porta hepatis, and common bile duct tissue samples were obtained surgically.

expression profiling was performed for extrahepatic bile duct tissues, including porta hepatis and common bile duct, to identify key regulatory genes. Quantitative reverse-transcription polymerase chain reaction (qRT-PCR) was conducted to confirm the changes in selected genes. Furthermore, we analyzed the relationship between differentially expressed genes in extrahepatic ducts tissue samples and prognosis of BA infants use a Spearman Nonparametric correlation analysis.

Materials and methods

Patients and samples

Between September 2013 and March 2014, 3 patients of type 3 BA were prospectively recruited from Children's Hospital, Fudan University. The tissue samples of porta hepatis, common bile duct and liver tissues were harvested surgically (**Figure 1**). During Kasai procedure, intraoperative cholangiography confirmed extrahepatic BA and tissue pathology revealed fibrosis mass in porta hepatic. However common bile duct was unaffected. Tissue samples were collected from another 24 BA patients for qRT-PCR validation. Clinical data were obtained retrospectively from clinical records (**Table 1**). The Ethics Committee of Children's Hospital, Fudan University approved our study protocol. The parents of all participants provided written informed consent prior to enrollment.

RNA extraction and microarray analyses

Total cellular RNA was isolated from fresh tissues, using an RNeasy Mini Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. Total RNA was quantified by NanoDrop ND-2000 (Thermo Scientific) and RNA integrity assessed with Agilent Bioanalyzer 2100 (Agilent Technologies). The sample labeling, microarray hybridization and washing were performed based on the manufacturer's instructions. Briefly total RNA was transcribed into double strand cDNA, synthesized into cRNA and labeled with Cyanine-3-CTP. The labeled cRNAs were hybridized onto microarray. After washing, the arrays were scanned by Agilent Scanner G2505C (Agilent Technologies).

Bioinformatics analysis of differentially expressed genes

Feature Extraction software (version10.7.1.1, Agilent Technologies) was used to analyze array images to acquire raw data. GeneSpring was employed for basic analysis of raw data. The raw data were normalized with the quantile algorithm. The probes that at least 100% of the values in any 1 out of all conditions with flags in "detected" were chosen for further analysis. Differentially expressed genes were then identified through fold change as well as P value calculated with t-test. The threshold set for up and down-regulated genes was a fold change ≥ 2.0 and a P value ≤ 0.05. Afterwards, GO analysis and pathway analysis were applied to determine the roles of these differentially expressed genes. Finally hierarchical clustering was performed to display the distinguishable patterns of gene expression among the samples.

Quantitative RT-PCR

Total cellular RNA was isolated from porta hepatis and common bile duct tissues with TRIzol reagent (Invitrogen, Carlsbad, CA, USA) and then reversely transcribed with PrimeScript RT reagent Kit with gDNA Eraser (Perfect Real Time) (TaKaRa, Dalian, China) in accordance with the manufacturer's instructions.

The expressions of selected up-regulated genes (IL7, VCAM1, CLDN2 and PLD1) and down-regulated genes (HAS2 and CADM3) were analyzed via qRT-PCR with a SYBR Green PCR kit (TaKaRa). The primers were listed in **Table 2**. The expression levels of genes were normalized to beta-actin (β-actin, ACTB) and calculated with the 2-ΔΔCt method (Livak & Schmittgen, 2001).

Table 1. Clinical characteristics of biliary atresia patients

Case	Age (d)	Gender	Diagnosis Type	ALT/AST (IU/L)	TB/DB (mmol/L)	GGT/ALP (IU/L)
1	53	F	Perinatal	29/107	129.1/86.7	1438/464
2	53	F	Perinatal	88/95	182.3/121.9	528/610
3	63	M	Perinatal	84/96	191.5/120.1	395/481
4	62	F	Perinatal	105/168	213.9/135.5	657/598
5	65	F	Perinatal	72/117	152/97.8	1079/388
6	81	M	Perinatal	53/89	168.8/109.6	1491/990
7	72	F	Perinatal	156/176	141.8/91.3	502/728
8	70	F	Perinatal	168/279	138.2/88.5	346/598
9	43	F	Perinatal	64/97	186/123.6	891/567
10	136	M	Perinatal	121/129	139.3/90.1	269/608
11	36	M	Perinatal	41/78	124.4/79.1	899/394
12	74	F	Perinatal	122/178	153.4/115.7	557/278
13	104	M	Perinatal	75/158	167.1/129.7	1254/554
14	74	M	Perinatal	88/143	133.9/83.	1144/892
15	75	F	Perinatal	128/117	142.6/95.9	1199/465
16	88	M	Perinatal	85/101	139.9/91.2	2208/526
17	60	F	Embryonic	117/314	202.5/129.3	448/470
18	83	M	Perinatal	180/240	162.9/103.2	2159/722
19	82	M	Perinatal	45/119	124.1/100.6	1090/298
20	47	F	Perinatal	68/106	133.5/85.2	861/796
21	75	M	Perinatal	83/107	140.9/95.2	232/512
22	58	M	Perinatal	67/108	190.2/120.3	131/574
23	58	F	Perinatal	55/117	199.2/129.6	428/756
24	72	M	Perinatal	58/77	122.3/80.2	693/355

TB: Total bilirubin; DB: Direct bilirubin; ALT: Alanine aminotransferase; AST: Aspartate aminotransferase; ALP: Alkaline phosphatase; GGT: γ-glutamyl transferase; case2, 7 and case 8 for microarray analysis, all cases for qRT-PCR validation.

Liver fibrosis score and follow-up research

Ishak liver fibrosis score was used to analyze the severity of fibrosis of liver specimens from 24 patients with BA. Score 0-2 was defined as mild fibrosis, 3-4 was defined as moderate fibrosis, 5-6 was defined as severe fibrosis. Elimination rate of jaundice (TB < 20 μmol/L) within 6 months after operation, the incidence of cholangitis were calculated. The diagnostic criteria for cholangitis included fever, increasing jaundice, acholic stools, with other causes of infection excluded. Follow-up data were obtained from our outpatient and inpatient referrals, as well as from interview by telephone or questionnaires.

Statistical analyses

All data were expressed as mean ± standard deviation. Statistical analysis was performed with Student's t-test for comparing two variables of microarray data. For example, the statistical significance of microarray result was analyzed by fold change. And a difference with $P < 0.05$ was considered statistically significant. The false discovery rate was also calculated to correct the P value. The threshold value for screening differentially expressed genes was a fold change ≥ 2.0 ($P < 0.05$). Furthermore, a differential expression of genes between porta hepatis and common bile duct tissues were analyzed with Student's t-test with SPSS (Version 19.0 SPSS, Chicago, IL, USA). The correlation between differentially expressed genes in liver tissue samples and prognosis of BA patients was analyzed with Spearman Nonparametric correlation analysis. $P < 0.05$ was considered statistically significant.

Results

Differentially expressed genes in extrahepatic duct tissues of BA

For profiling differentially expressed genes in extrahepatic duct tissues of BA, genome-wide analysis was performed for fibrosis mass in porta hepatis and common bile duct tissues. Using the authoritative data sources, the gene expression profiles were assessed in porta hepatis versus common bile duct tissues (self-control). It was found that 140 genes were differentially expressed (fold change ≥ 2.0, $P < 0.05$) between fibrosis mass in porta hepatis and common bile duct tissues. Among them, 19 genes were up-regulated (> 2 folds in porta hepatis vs. common bile duct) and 121 genes down-regulated (fold change ≥ 2.0, $P < 0.05$).

Construction of co-expression network using GO and pathway analyses

As revealed by GO analysis, these up-regulated and down-regulated genes were associated with 49 gene GO terms corresponding to transcripts, i.e., heterophilic cell-cell adhesion composed of 4 targeted genes (recommended *P*-value < 0.05) (**Figure 2A**).

Table 2. Sequences of primers in selected genes used in qRT-PCR validation

Gene	Sequence (5'-3')	length (bp)	Product length (bp)
HAS2	TGGCTAAACCAGCAGACCCG	20	140
	GTGGCAATGAGAAAGAAAGGAAAG	24	
IL7	GCTCCAGTTGCGGTCATCAT	20	150
	GTTTGCCATCTTTACCTTCA	20	
VCAM-1	TGACCTTCATCCCTACCATT	20	110
	TGTTTGCGTACTCTGCCTTT	20	
CLDN-2	GGCTCCAGTGGGTGTTTCTA	20	150
	GCTTGGTGCTATGGTCTTTCA	21	
CADM3	ACCAACTGCGATGATTAGGC	20	115
	CTCCTTCTCCCATAGGTACTGC	22	
PLD1	GGGAAAGCGTGACAGTGAAATG	22	147
	TCATCAAGATAGCCAAGGACAACC	24	
β-actin	TGGCACCACACCTTCTACAATG	22	166
	ATAGCACAGCCTGGATAGCAAC	22	

And pathway analysis indicated that these genes might target 22 gene pathways corresponding to transcripts, i.e., cell adhesion molecules (CAMs) composed of 6 targeted genes and protein digestion & absorption composed of 4 targeted genes (**Figure 2B**).

Subsequently, we constructed a co-expression network of genes including differentially expressed genes. Our data showed that the co-expression network was composed of 14 regulatory genes. Among these genes, phospholipase D1 (PLD1) might play a critical regulatory role in this co-expression network (**Figure 3**).

Validation of selected differentially expressed genes via qRT-PCR

Four up-regulated genes of interleukin 7 (IL7), vascular cell adhesion protein 1 (VCAM1), claudin-2 (CLDN2), PLD1 and 2 down-regulated genes of hyaluronan synthase 2 (HAS2) and cell adhesion molecule 3 (CADM3) were randomly selected for verification in these 24 BA patients.

The results showed that the expressions of IL7, VCAM1, CLDN2 and PLD1 were over-regulated while HAS2 and CADM3 down-regulated in porta hepatis tissues relative to self-contrast common bile duct counterparts ($P < 0.05$; **Figure 4A**, **4B**).

Correlation between differentially expressed genes in extrahepatic ducts tissue samples and prognosis of BA patients

To explore whether differentially expressed genes were associated with the severity of liver fibrosis of BA patients, we performed a correlation analysis. We found that the expression level of VCAM1 was positively correlated with severity of liver fibrosis in the BA infants ($r = 0.590$, $P = 0.002$, **Figure 5**). To address whether differentially expressed genes were associated with the prognosis of BA patients, we performed a 6-month follow-up study. Six months after operation, The VCAM1 expression level was significantly higher in patients with their jaundice not eliminated than in those with their jaundice eliminated ($r = 0.501$, $P = 0.013$). However, no significant difference in these mRNA expressions of differentially expressed genes was found between patients with cholangitis reoccurred 2 or more times than in those with no cholangitis recurred.

Discussion

Up to the present, the etiology and pathogenesis of BA have been ill-defined. Several potential molecular mechanisms, including genetic susceptibility, viral infection, toxic insults and immune-mediated injury, were often considered to be independent or co-existing risk factors [19, 20]. Some studies of BA involved DNA microarrays [14, 21]. Bezerra et al used gene microarrays for identifying differentially expressed genes in liver samples of infants of BA and found that genetic induction of proinflammatory immunity might play a key role in BA. There was a coordinated activation of genes involved in lymphocyte differentiation. Among these genes, the overexpression of osteopontin and interferon 1 implicated a potential role of Th-1-like cytokines in disease pathogenesis [7].

Recently mounting evidence has shown that genetic susceptibility was an important factor in the pathogenesis of BA [14, 16-18, 22]. Zhang et al found that embryonic and perinatal forms of BA could be distinguished by gene expression profiling [16]. And the regulatory

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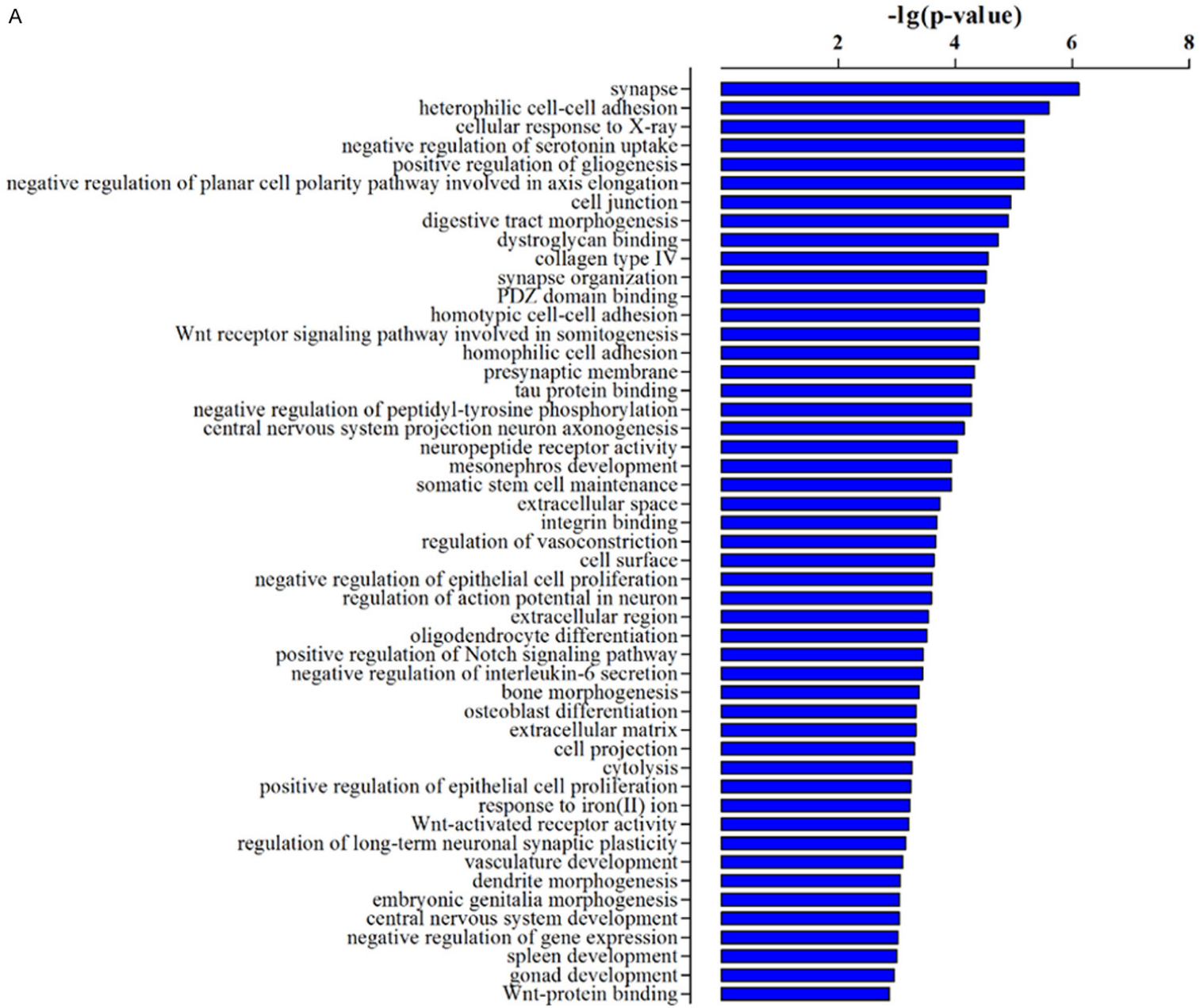
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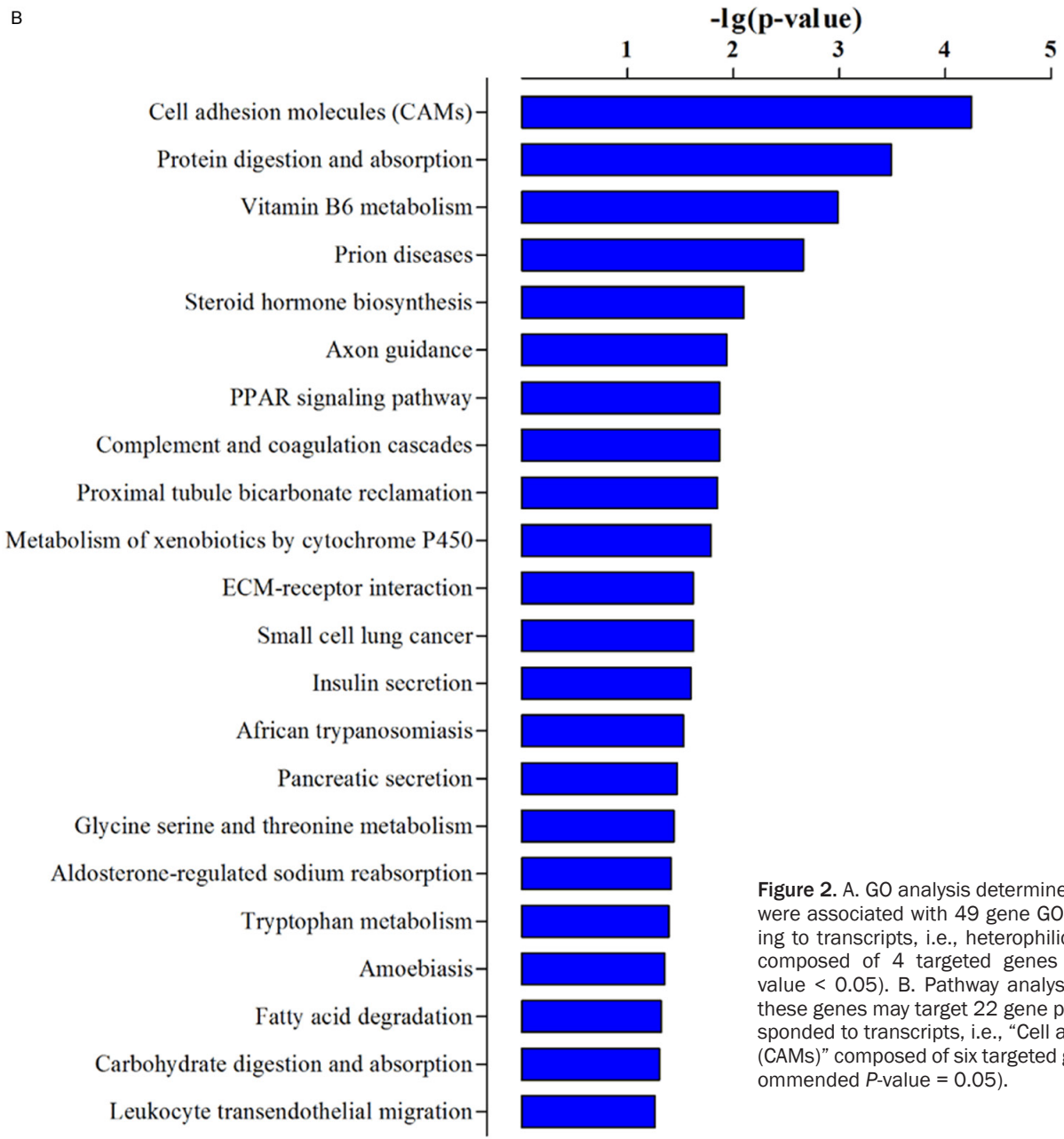

Figure 2. A. GO analysis determined that these genes were associated with 49 gene GO terms corresponding to transcripts, i.e., heterophilic cell-cell adhesion composed of 4 targeted genes (recommended *P*-value < 0.05). B. Pathway analysis determined that these genes may target 22 gene pathways that corresponded to transcripts, i.e., "Cell adhesion molecules (CAMs)" composed of six targeted genes (with the recommended *P*-value = 0.05).

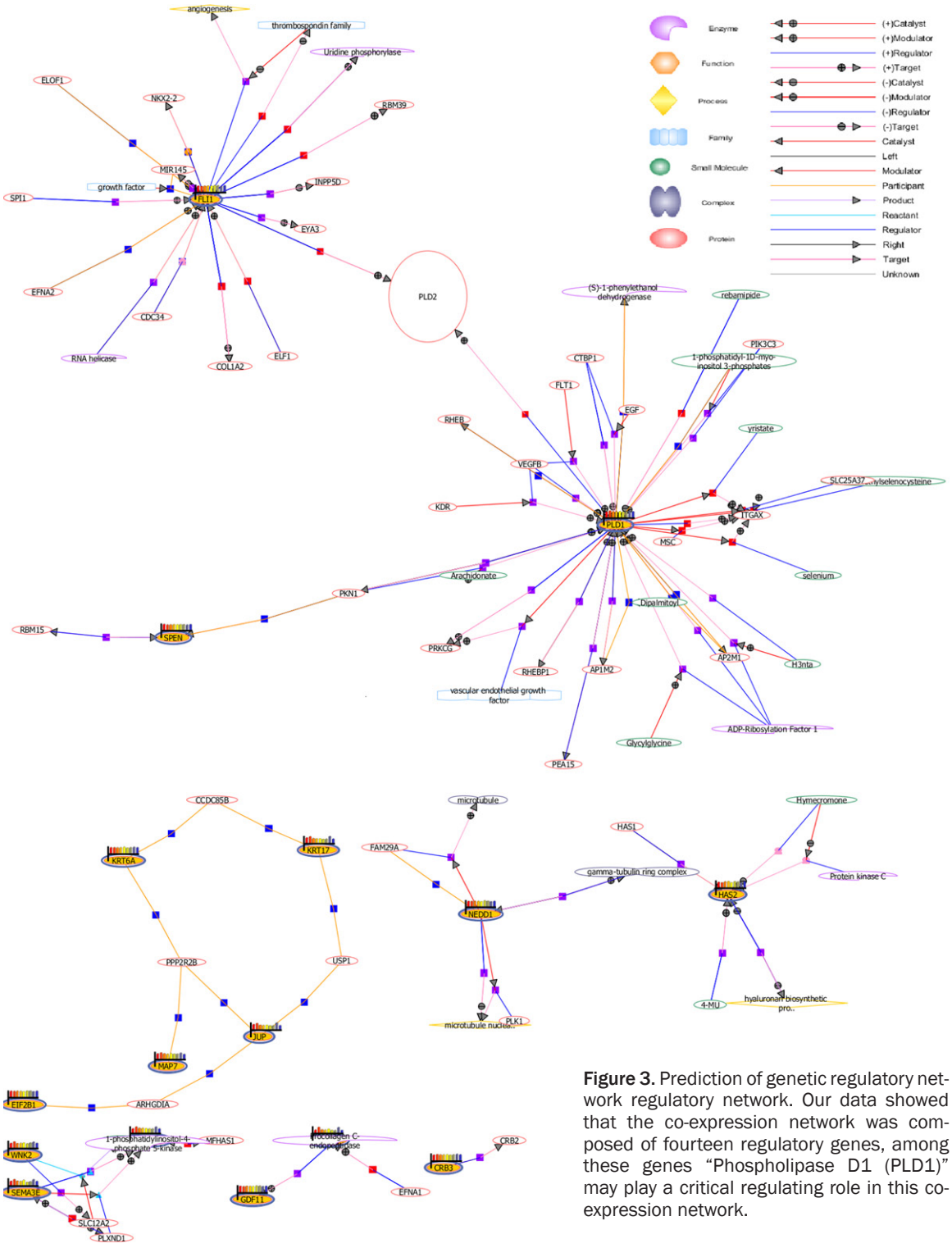

Figure 3. Prediction of genetic regulatory network regulatory network. Our data showed that the co-expression network was composed of fourteen regulatory genes, among these genes "Phospholipase D1 (PLD1)" may play a critical regulating role in this co-expression network.

genes were predominantly represented in the embryonic form (45% of genes) with an unique pattern of expression of genes involved in chromatin integrity/function (Smarca-1, Rybp & Hdac3) and an uniform overexpression of 5 imprinted genes (Igf2, Peg3, Peg10, Meg3 & IPW). It suggested a failure of down-regulating embryonic gene programs [16]. Petersen et al employed gene microarrays for identifying differentially expressed genes in an infective murine model for BA. Most up-regulated genes in BA-positive mice encoded proinflammatory

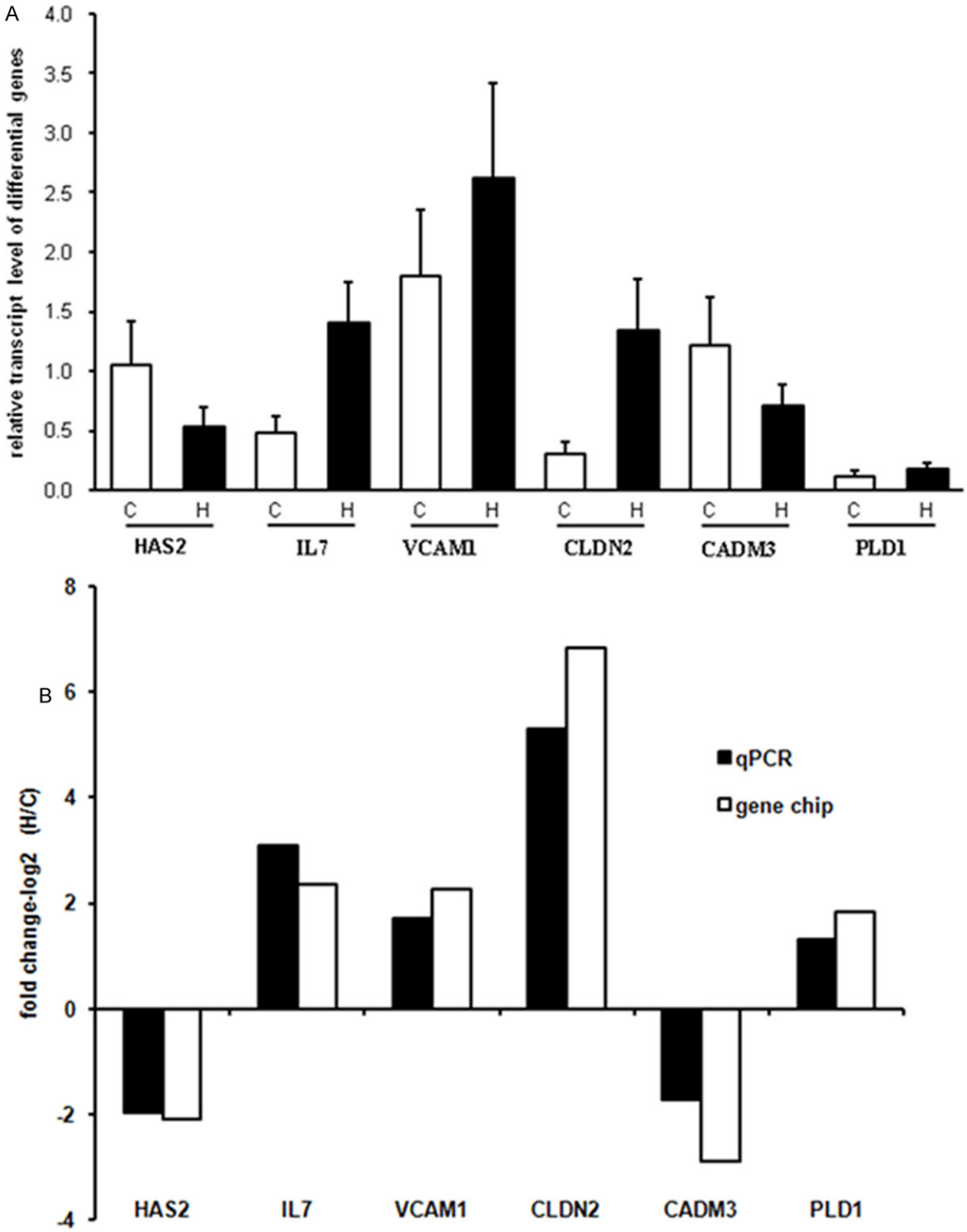

Figure 4. A, B. qRT-PCR validation of some differentially expressed genes in 24 BA tissue samples. The data showed that expressions of IL7, VCAM1, CLDN2 and PLD1 were over-regulated, and HAS2 and CADM3 were downregulated in porta hepatis tissue samples relative to the self-contrast Common bile duct tissues ($P < 0.05$). Note: C mean common bile duct, H mean Porta hepatis.

cytokines involved in the Th1 pathway, such as CCL2, CCL5, CCR5, CXCL10, CCL2, IL1F5 and DDR3 and granzymes A and B in apoptosis. And TIMD2 played a critical role in the regulation of a Th2-type response through an inhibition of interferon gamma [17].

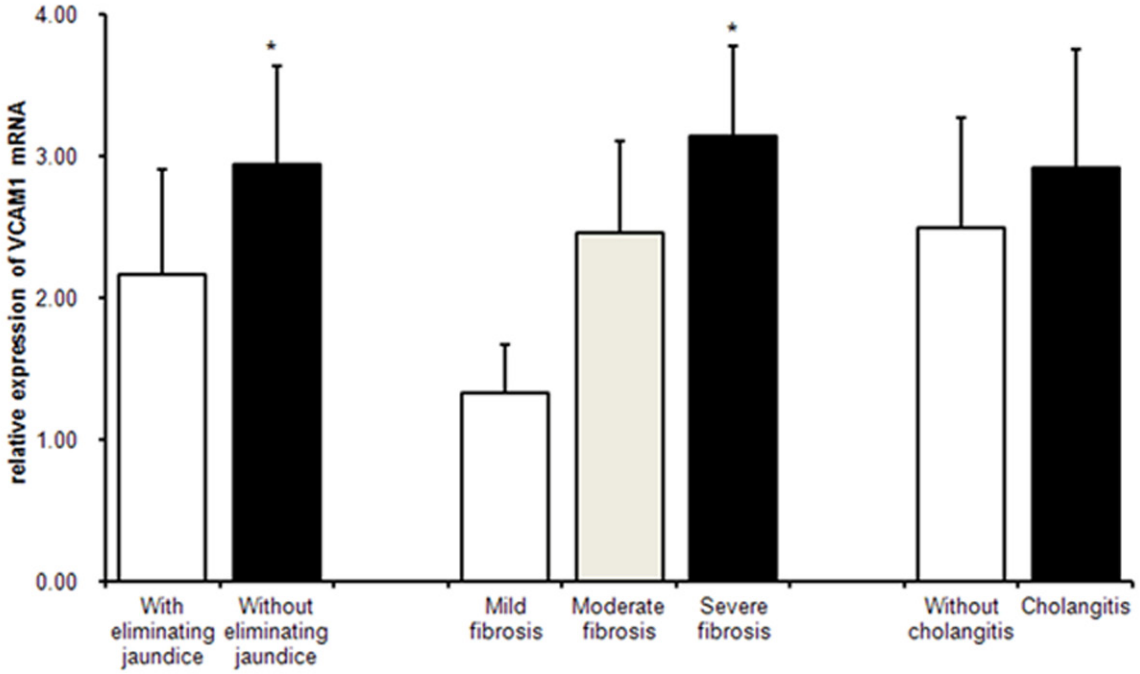

Figure 5. Correlation between expression of VCAM1 in liver tissue samples and prognosis of biliary atresia patients within 6 months.

Up until now, there has been no report of identifying genes for the pathogenesis of fibrosis in porta hepatis with BA. The data of the present study was the first to show that a total of 140 genes were differentially expressed between fibrosis mass in porta hepatis and common bile duct tissues with fold changes of 2 or more. It was found that 19 genes were up-regulated (over 2 folds in porta hepatis vs. common bile duct) and 121 genes down-regulated. GO and pathway analyses predicted that down-regulated and up-regulated transcripts of genes were associated with cellular process (ontology: biological process), cell (ontology: cellular component) and binding (ontology: molecular function) associated with 22 gene pathways corresponding to transcripts, i.e., "cell adhesion molecules (CAMs)" composed of 6 targeted genes (recommended *P*-value = 0.05) and protein digestion and absorption. The former appeared to be involved in the pathogenesis of liver fibrosis with BA [23]. Therefore it might play a key role in the pathogenesis of fibrosis in porta hepatis with BA.

Subsequently a co-expression network was constructed for these differentially expressed genes. Among 14 regulatory genes, phospholipase D1 (PLD1) played a critical regulatory role. And one recent study indicated that phospholipase D1 played an important role in the development and progression of liver fibrosis in rats [24]. In addition, the presence was confirmed for some of these differentially expressed genes. The results showed that IL7, VCAM1, CLDN2 and PLD1 were up-regulated while HAS2 and CADM3 down-regulated in porta hepatis tissue samples as compared with control common bile duct tissues. The data of the present study demonstrated that the expressions of these altered genes could contribute to fibrosis in porta hepatis with BA. Further study may provide useful insights into the mechanisms of BA.

Finally we found that the expression level of VCAM1 was positively correlated with severity of liver fibrosis in the BA infants. Six months after operation, The VCAM1 expression level was significantly higher in patients with their jaundice not eliminated than in those with their jaundice eliminated. Consequently, it is reasonable to speculate that VCAM1 may play an important role in the pathogenesis of liver fibrosis development in BA infants. Interestingly, among these differentially expressed genes, HAS2 was associated with the pathway of cell adhesion molecules. As a constituent of extra-

cellular matrix, hyaluronic acid (HA) is a high-molecular-weight non-branched polysaccharide synthesized by a wide variety of organisms from bacteria to mammals. It is actively produced during wound healing and tissue repair to provide a framework for an in-growth of blood vessels and fibroblasts [25]. As a member of newly identified vertebrate gene family, HAS2 encodes putatively hyaluronan synthases [26-28]. Some studies have found that inhibiting HA synthesis modulates TGF beta1-dependent responses in these cells and arresting the differentiation of fibroblast into myofibroblast [29]. And other studies also proved that HAS2 was associated with fibrosis [30, 31].

In summary, the pathogenesis of BA has remained elusive. Many genes with altered expression in BA were related with fibrosis in porta hepatis. However, it is possible that these genes may also play a role in fibrosis in porta hepatis of patients with BA. Further studies are needed for fully understanding this disease.

Acknowledgements

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Disclosure of conflict of interest

None.

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