

# Bioinformatics-based identification of key pathways and candidate genes for estrogen-induced intrahepatic cholestasis using DNA microarray analysis

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**Abstract.** Estrogen-induced intrahepatic cholestasis (EIC) has increased incidence during pregnancy, and within women taking oral contraception and postmenopausal hormone replacement therapy. However, the pathology underlying EIC is not well understood. The aim of the present study was to identify key pathways and candidate genes in estrogen-induced intrahepatic cholestasis (EIC) that may be potential targets for diagnosis and treatment. A whole-genome microarray (4x44K) analysis of a 17 $\alpha$ -ethinylestradiol (EE)-induced EIC rat liver model was performed. Bioinformatics-based methods were used to identify key pathways and candidate genes associated with EIC. The candidate genes were validated using a reverse transcription quantitative polymerase chain reaction assay. A total of 455 genes were differentially expressed ( $P < 0.05$  and fold change  $> 2.0$ ) following EE treatment, including 225 down-regulated genes and 230 upregulated genes. Sulfotransferase family 1E member 1, cytochrome P450 family 3 subfamily A member 2, carbonic anhydrase 3, leukotriene C4 synthase and ADAM metalloproteinase domain 8 were the 5 candidate genes identified to be differentially expressed and involved in the metabolism of estrogens and bile acids and the regulation of inflammation and oxidative stress. The Analyses of Gene Ontology enrichment, Kyoto Encyclopedia of Genes and Genomes pathways and protein-protein interaction network associated-modules identified several key pathways involved in the homeostasis of lipids and bile acids and in AMPK, p53 and Wnt signaling. These key pathways and candidate genes may have critical roles in the pathogenesis of EIC. In

addition, reversing the abnormal expression of candidate genes or restoring the dysfunction of key pathways may provide therapeutic opportunities for patients with EIC.

## Introduction

Intrahepatic cholestasis leads to systemic and intrahepatic retention of toxic bile acids that initially cause liver injury and subsequently result in biliary fibrosis and cirrhosis (1). Estrogens and their metabolites are well-known causes of estrogen-induced intrahepatic cholestasis (EIC) during pregnancy and in women taking oral contraception and postmenopausal hormone replacement therapy (2). Its pathogenesis arises primarily through genetic predisposition but also to a lesser extent by environment and immunology factors (3). Intrahepatic cholestasis of pregnancy (ICP) is the most common type of EIC, with an estimated prevalence of 0.4-1.5% worldwide (4). Delayed diagnosis of ICP may increase the risks of stillbirth and early delivery (5). However, the pathogenic and physiopathological mechanisms of EIC are not well understood. EIC is currently treated with ursodeoxycholic acid (UDCA), but 30-50% of patients do not respond to UDCA treatment (6). Therefore, identification of key pathways and candidate genes for EIC may provide a greater understanding of this disease and aid in the development of novel drug targets.

Estrogen levels increase significantly during pregnancy and following administration of oral contraception and postmenopausal replacement therapy, particularly in patients with EIC (7). High estrogen levels induce acute cholestasis by impairing both bile flow and bile acid metabolism, causing downstream dysfunction of bile acid homeostasis (8). Accordingly, toxic bile acids accumulate in the liver, inducing oxidative stress and inflammatory reactions and the onset of liver injury (9). Clinical studies have indicated that oxidative stress responses are amplified in ICP patients compared to healthy pregnant women (10,11). In addition, pro-inflammatory cytokines, including interleukin (IL)-6 and IL-17, are markedly increased in patients with ICP compared with healthy pregnant women (12,13). Animal models of EIC have also demonstrated high expression levels of pro-inflammatory cytokines and an increased oxidative stress response (14,15).

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The development of bioinformatics has provided new insights into the diagnosis and treatment of cholestatic liver diseases (16–18). Using integral microRNA (miRNA) and mRNA microarrays, Nakagawa *et al* (16) identified that the miR-425-mediated T cell receptor signaling pathway had an important role in regulating the synthesis of inflammatory cytokines in CD4<sup>+</sup> T cells of patients with primary biliary cholangitis (PBC). Suppression of the T cell receptor signaling-associated regulator or miR-425 has therefore been suggested to be a promising immunotherapeutic strategy against PBC (16). Wang *et al* (17) identified serpin family E member 1 (*PAI-1*) as the key gene in mice with extrahepatic cholestasis following bile duct ligation, and demonstrated that inhibiting *PAI-1* may attenuate cholestatic liver diseases. These studies suggest that bioinformatics is a useful strategy to identify key pathways and candidate genes involved in disease as a foundation to develop new therapies. However, to the best of our knowledge, a comprehensive bioinformatical analyses of the cellular and molecular mechanisms underlying EIC has not been performed.

Cholestasis induced by 17 $\alpha$ -ethinylestradiol (EE) is a rodent model widely used to investigate the molecular mechanisms of EIC (19). The present study aimed to identify key pathways and candidate genes using a whole-genome microarray (4x44K) to analyze an EE-induced cholestatic rat model. The most significant differentially expressed genes (DEGs) involved in the metabolism of estrogen and bile acids and the regulation of inflammation and oxidative stress were shortlisted as candidate genes. In addition, Gene Ontology (GO), Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway enrichment and protein-protein interaction (PPI) network analyses were performed to identify key pathways involved in EIC. A total of 5 candidate genes and several key pathways were identified, which may be candidate diagnostic biomarkers or therapeutic targets for EIC.

## Materials and methods

**Animals and treatments.** A total of 12 male Sprague Dawley rats weighing 200 $\pm$ 20 g were obtained from the Center of Experimental Animals of Hubei Province. All animals acclimated to the laboratory conditions for 1 week prior to the experiment and were kept at 25 $\pm$ 2°C with a 12:12 h light:dark cycle. Animals had *ad libitum* access to standard laboratory chow and tap water. The present study was approved by the Ethical Committee on Animal Experimentation of Tongji Medical College, Huazhong University of Science and Technology (Wuhan, China). Animals were randomly divided into control and model groups (n=6 per group). Control and model rats were administered subcutaneous (s.c.) propylene glycol (solvent of EE, 0.25 ml/100 g, s.c.) or EE (5 mg/kg, s.c.) for 5 consecutive days, respectively. Rat conditions and body weight were recorded every day. Rats were fasted overnight following the last treatment prior to sacrifice. Bile flow was measured according to a previously described method (14). Blood samples and liver tissues were collected for subsequent analysis.

**Biochemical determinations and histological analyses.** Serum aspartate aminotransferase (ALT), alanine aminotransferase

(AST), alkaline phosphatase (ALP) and total bile acids (TBA) were analyzed using commercial kits (Nanjing Jiancheng Bioengineering Institute) according to the manufacturer's protocol. A section of the same liver parts from each rat was dissected and fixed in 10% formalin for 24 h in a room temperature, washed, dehydrated in alcohol gradients (100, 100, 95, 80 and 75%, 2 min each) and paraffin-embedded. Each slide was subsequently stained with hematoxylin (0.2%, 5 min, room temperature) and eosin (0.5%, 30 sec, room temperature), and then assessed histologically with a light microscope (magnification, x200, EVOS FL Auto; Thermo Fisher Scientific, Inc.).

**Gene expression analyses.** Total RNA of 3 randomly selected rat liver tissues were extracted using RNAiso Plus (Takara Bio, Inc.) and purified using an RNeasy mini kit (Qiagen GmbH) following the manufacturer's instructions. Total RNA was amplified and labeled with an Agilent Quick Amp Labeling kit (Agilent Technologies, Inc.). Each slide containing rat liver total RNA was hybridized with an Agilent Whole Rat Genome Oligo Microarray (4x44K) for 17 h. Slides were then washed and scanned using an Agilent DNA Microarray Scanner (Agilent Technologies, Inc.). Data was extracted with Agilent Feature Extraction software v10.7 (Agilent Technologies, Inc.) and subsequently filtered for detection of normalization and significant changes, using P<0.05 as the cut-off value, and DEGs compared to the control rats, using fold change >2 or <0.5 as the cut-off. The heatmap of DEGs was analyzed by SBC analysis system (Shanghai Biotechnology Corporation). Technical support was provided by the Shanghai Biotechnology Corporation.

**Reverse transcription quantitative polymerase chain reaction (RT-qPCR).** Total RNA of all rat liver samples was isolated using TRIzol<sup>®</sup> reagent (Thermo Fisher Scientific, Inc.) and then reverse transcribed into cDNA using the PrimeScript<sup>™</sup> RT Master Mix (Takara Biotechnology Co., Ltd.). The RT steps were: 37°C, 15 min and 85°C, 5 sec. Each cDNA was used as a template for RT-qPCR amplification, performed using SYBR Premix Ex Taq<sup>™</sup> (Takara Biotechnology Co., Ltd.) and pairs of forward/reverse primers for the candidate genes and rat  $\beta$ -actin gene. The qPCR steps were: initial denaturation, 95°C, 30 sec; PCR reaction, 95°C, 5 sec, 60°C, 34 sec, 40 cycles. Independent reactions were performed in triplicate using an Applied Biosystems StepOnePlus Real-time PCR system (Applied Biosystems). Threshold cycle (C<sub>q</sub>) values were normalized to those for  $\beta$ -actin ( $\Delta$ C<sub>q</sub>). The 2<sup>− $\Delta$ C<sub>q</sub></sup> method was used to calculate the relative changes of gene expression (20). The primer sequences of candidate genes and  $\beta$ -actin are presented in Table I.

**Functional enrichment analyses of the DEGs.** Functional annotation of DEGs was performed using the online Database for Annotation, Visualization, and Integrated Discovery (DAVID; <https://david.ncifcrf.gov/>). DAVID provides comprehensive functional annotations tools for the analysis of the genes and proteins included in the database, for users to obtain biological information. The functions of the DEGs was determined via GO terms and KEGG pathway enrichment using DAVID, with P<0.05 used as the cut-off for statistical significance.

Table I. Sequence of primers for 6 candidate genes and  $\beta$ -actin for reverse transcription quantitative polymerase chain reaction.

Gene name	Direction	Sequence (5'-3')
Sult1e1	Forward	ATTCAATGCTGCCAGAGACC
	Reverse	TCATTTGCTGCTGGTAGTGC
Cyp3a2	Forward	TGACTGCTCTTGATGCATGGTT
	Reverse	ATCACAGACCTTGCCAACTCCT
Car3	Forward	GCTCCTTTTAATCACTTCGACC
	Reverse	AGCCACACAATGCACTCCTC
Ltc4s	Forward	GAAGAACTTTCCACGTGTCG
	Reverse	GTGCAGCCATTGCCACTAGC
Adam8	Forward	GCCTCGGACCTTAGAAAT
	Reverse	GGCATAACGGCTGATGTA
$\beta$ -actin	Forward	CACCCGCGAGTACAACCTTC
	Reverse	CCCATACCCACCATCACACC

Sult1e1, sulfotransferase family 1E member 1; Cyp3a2, cytochrome P450 family 3 subfamily A member 2; Car3, carbonic anhydrase 3; Ltc4s, leukotriene C4 synthase; Adam8, ADAM metalloproteinase domain 8.

**Construction of PPI networks and analysis of modules.** The Search Tool for Retrieval of Interacting Genes and Proteins (STRING) database (v10.0; <http://string-db.org/>) was used for PPI network construction of the DEGs. Genes without associations with other genes were removed. Interactions with a combined score >0.4 were considered significant. PPI networks were used for module screening using the Molecular Complex Detection (MCODE) in Cytoscape (v3.6.0) bioinformatics integration platform (21). Significant modules were those with scores >3 and nodes >4. KEGG pathway enrichment analysis for DEGs was performed in each module using ClueGO software (version 2.5.0, Institute for Genomics and Bioinformatics Graz University of Technology, Graz, Austria).

**Statistical analyses.** Statistical analyses were performed using SPSS software v13.0 (SPSS Software, Inc.). All data are presented as the mean  $\pm$  standard deviation. A Student's t-test was used to compare the differences between two groups.  $P < 0.05$  was considered to indicate a statistically significant difference.

## Results

**EE-induced cholestasis and liver injury.** The body weight of the control rats gradually increased, while that of model rats initially increased, but then fell. A significant difference ( $P < 0.01$ ) in body weight between control and model rats was observed at days 5 and 6 (Fig. 1A). Bile flow was suppressed ( $P < 0.01$ ) in the EE-treated rats (Fig. 1B). Serum ALT, AST, ALP and TBA levels were significantly increased in the EE-induced rats compared with the control rats (Fig. 1C). Histological evaluation of the hepatic tissues indicated that control rats exhibited normal structures, while there was evidence of neutrophil infiltration, edema and hepatic necrosis

Table II. Top 5 downregulated and upregulated differentially expressed genes (n=3).

Direction	Symbol	Log <sub>10</sub> FC	P-values
Downregulated	Car3	-1.988	0.0019
	Sult1e1	-1.966	0.0272
	Cyp3a2	-1.531	0.0322
	LOC100912610	-1.444	0.0277
	Ltc4s	-1.396	0.0042
Upregulated	Rasd2	1.894	0.0034
	Adam8	1.882	0.0074
	Pvalb	1.859	0.0153
	Slc5a1	1.705	0.0010
	Cited4	1.638	0.0009

DEGs, differentially expressed genes; FC, fold change; Sult1e1, sulfotransferase family 1E member 1; Cyp3a2, cytochrome P450 family 3 subfamily A member 2; Car3, carbonic anhydrase 3; Ltc4s, leukotriene C4 synthase; Rasd2, RASD family member 2; Adam8, ADAM metalloproteinase domain 8; Pvalb, parvalbumin; Slc5a1, solute carrier family 5 member 1; Cited4, Cbp?P300 interacting transactivator with Glu/Asp rich carboxy-terminal domain 4.

in model rats (Fig. 1D). These results were in concordance with the previously published data (22) and thereby suggest that EE induced cholestasis and liver injury in rats.

**Identification of DEGs and candidate genes.** Following preprocessing of the microarray data, the gene expression data were normalized and visualized with a box plot. A good normalization performance was obtained (Fig. 2A). In total, 455 DEGs were identified, including 225 downregulated genes and 230 upregulated genes. The heatmap function was used to observe DEGs in different samples (Fig. 2B). The top 5 downregulated and upregulated DEGs are listed in Table II. Genes were thereafter shortlisted as candidate genes based on evidence of their involvement in the metabolism of estrogen and bile acids or in the regulation of inflammatory reaction and oxidative stress, either from the literature or the data from the present study. These were sulfotransferase family 1E member 1 (*Sult1e1*), cytochrome P450 family 3 subfamily A member 2 (*Cyp3a2*), carbonic anhydrase 3 (*Car3*), leukotriene C4 synthase (*Ltc4s*) and ADAM metalloproteinase domain 8 (*Adam8*). To confirm the reliability of DNA microarray data, the mRNA expression levels of these candidate genes were investigated by RT-qPCR. The results were consistent with those from the DNA microarray data (Fig. 2C).

**Functional enrichment analysis for gene expression.** To explore the functions of DEGs, GO and KEGG pathway enrichment analyses were performed using the DAVID. As demonstrated in Fig. 3, analysis of GO indicated that the upregulated genes were primarily enriched in the regulation of protein binding, calcium ion binding, epithelial cell differentiation and extracellular exosome, while the downregulated genes were primarily enriched in regulators of iron ion binding, heme binding, aromatase activity and oxidation-reduction.

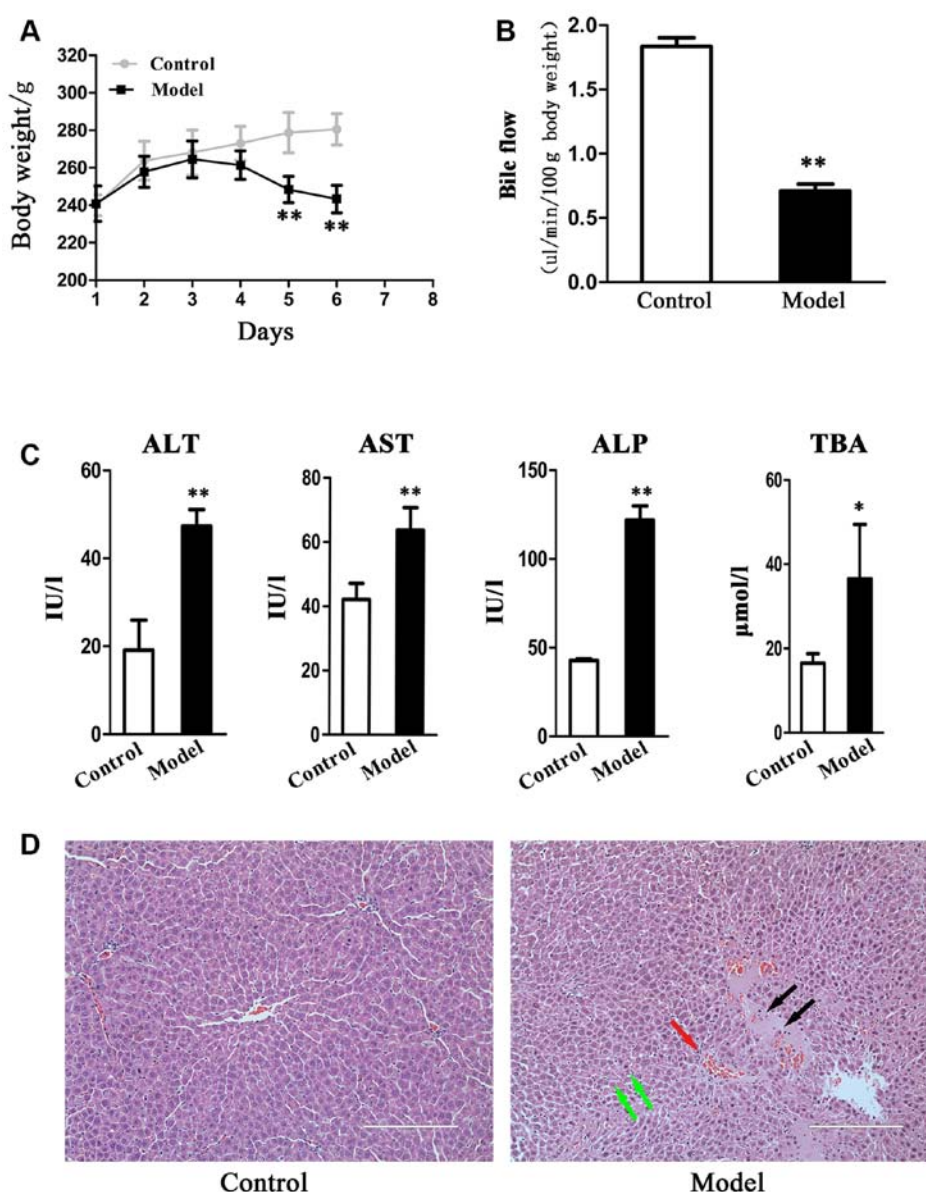


Figure 1.  $17\alpha$ -ethinylestradiol-induced cholestasis and liver injury. (A) Changes in body weight and (B) bile flow in EIC rats. Bile was collected in a 10-minute period to monitor the flow. (C) Serum biochemical index of AST, ALT, ALP and TBA. (D) The images of representative hematoxylin and eosin stained liver sections (magnification,  $\times 200$ ). Liver necrosis, neutrophil infiltration and edema are marked by black, red, and green arrows, respectively. Data are presented as the mean  $\pm$  standard deviation ( $n=6$ ). \* $P<0.05$  and \*\* $P<0.01$  vs. control. EIC, estrogen-induced intrahepatic cholestasis; AST, aspartate aminotransferase; ALT, alanine aminotransferase; ALP, alkaline phosphatase; TBA, total bile acids.

KEGG analysis was used for the identification of significantly enriched pathways of the DEGs. The upregulated DEGs were primarily enriched in the pathways of 5'AMP-activated protein kinase (AMPK) and prolactin and those involved in protein digestion, protein absorption and fatty acid metabolism. The downregulated DEGs were primarily enriched in steroid hormone biosynthesis, retinol metabolism, metabolic pathways and arachidonic acid metabolism. A total of 3 pathways also enriched in the downregulated DEGs were involved in regulating bile acid homeostasis, including bile secretion, peroxisome proliferator-activated receptor (PPAR) signaling and primary bile acid biosynthesis.

**PPI network construction and module selection.** Protein interactions of DEGs were predicted by the STRING database and then the PPI networks were constructed for those with a

combined protein pair score  $>0.4$  (Fig. 4). Modules of genes in the PPI network were identified by the MCODE plugin in Cytoscape. The top 4 significant modules were selected, and the cellular pathways of the genes involved in these modules were analyzed using ClueGO. As demonstrated in Fig. 5, the cellular pathways were involved in steroid hormone biosynthesis, retinol metabolism, arachidonic acid metabolism, bile secretion, and p53 and Wnt signaling.

## Discussion

EIC is characterized by high estrogen levels, accumulation of toxic bile acids, hepatic inflammation and oxidative stress, which leads to the fibrosis, cirrhosis and eventually failure of the liver (23). Liver transplantation remains the only strategy for patients with the end-stage EIC (24). For EICs detected at

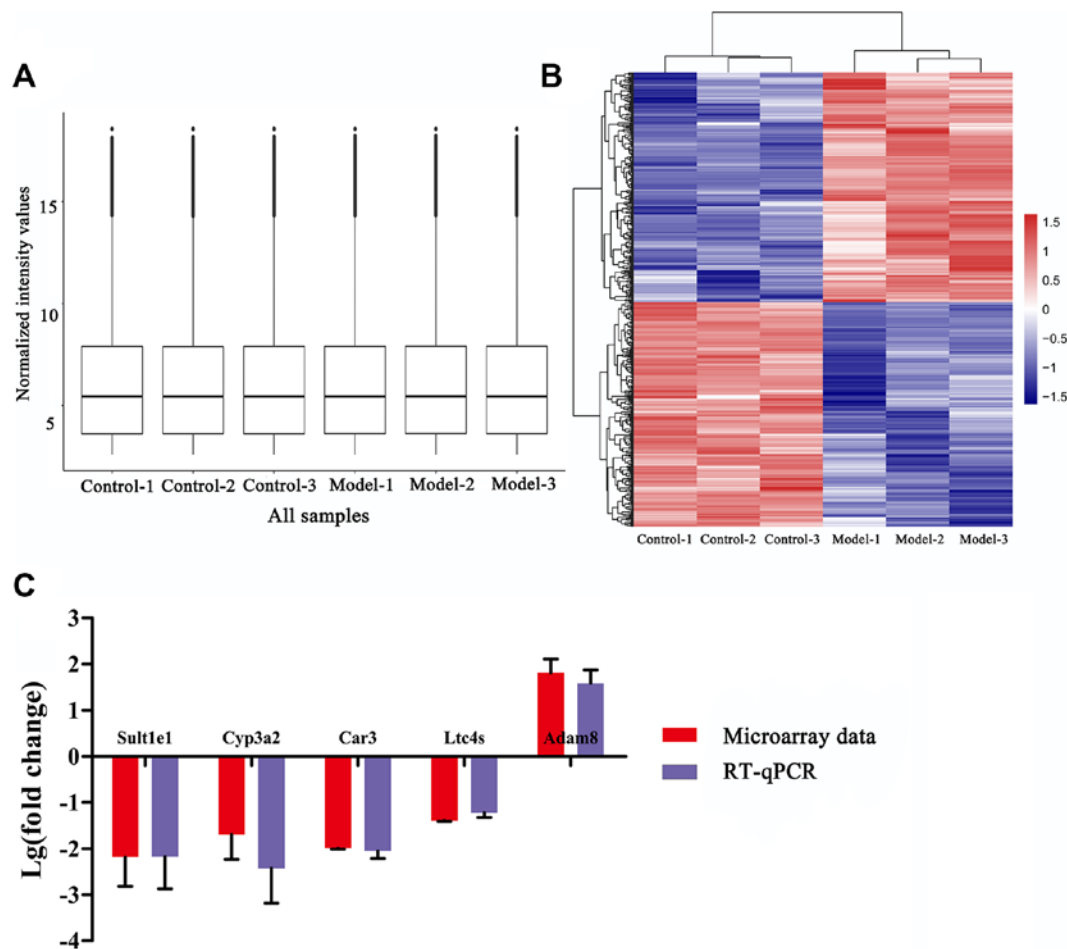


Figure 2. Cluster analysis of DEGs and a selection of candidate genes. (A) Box plot for normalized gene expression data. The medians are similar, indicating a high level of normalization. (B) Cluster analysis of DEGs is exhibited in a heat map ( $n=3$ ). Red indicates upregulated expression, while blue indicates down-regulated expression. (C) RT-qPCR confirmation for the 5 candidate genes ( $n=6$ ). Each column represents the  $\log_{10}$  fold change of candidate genes normalized to  $\beta$ -actin. The data are presented as the mean  $\pm$  standard deviation. DEGs, differentially expressed genes; RT-qPCR, reverse transcription quantitative polymerase chain reaction; Sult1e1, sulfotransferase family 1E member 1; Cyp3a2, cytochrome P450 family 3 subfamily A member 2; Car3, carbonic anhydrase 3; Ltc4s, leukotriene C4 synthase; Adam8, ADAM metallopeptidase domain 8.

earlier stages, UDCA and obeticholic acid, a nuclear receptor agonist, are the first- and second-line drugs; however, their curative effects are limited (25). In addition, the mechanisms involved in the pathogenesis and physiopathology of EIC are not well understood. Understanding the molecular mechanisms involved in the development and progression of EIC is therefore crucial for developing and evaluating diagnostic and management strategies.

In the present study, a whole-genome microarray was used to identify key pathways and candidate genes within EE-induced cholestatic rats. The results identified a total of 455 DEGs, including 225 downregulated genes and 230 upregulated genes. Among the top upregulated and downregulated genes, those involved in the metabolism of estrogens and bile acids, and the regulation of inflammation and oxidative stress, were identified and validated as candidate genes. These were *Sult1e1*, *Cyp3a2*, *Car3*, *Ltc4s* and *Adam8*. The elevated estrogen levels that are typically observed in patients with EIC and animal models induce dysfunction of bile acid hemostasis through activating estrogen-responsive receptors, leading to the accumulation of toxic bile acids in the liver and the development of liver

injury (1). A phase II drug-metabolizing enzyme, *Sult1e1*, is known to catalyze the sulfoconjugation of estrogens and their metabolites (26). It has been demonstrated *Sult1e1* is down-regulated during several liver diseases, including cholestasis, and this is associated with elevated serum estrogen (27,28). An additional study investigating livers in a cystic fibrosis mouse model indicated that elevated hepatic *Sult1e1* levels may result in decreased levels of estrogens and decreased expression of estrogen-responsive receptors (29). *Cyp3a2*, a human ortholog of cytochrome P450 family 3 subfamily A member 4 (CYP3A4), is a phase I detoxifying cytochrome P450 enzyme that catalyzes the elimination of bile acid (30) and thereby decreases toxic bile acid levels in the liver (31). Numerous studies have demonstrated that approved drugs including rifampicin, phenobarbital and bezafibrate protect against cholestasis through inducing CYP3A4 activity and detoxifying bile acid levels (32-34). In the present study, the extremely low expression of *Sult1e1* and *Cyp3a2* may limit the metabolism of estrogens and bile acids and lead to their accumulation in the liver. Therefore, reversing the expression of *Sult1e1* and *Cyp3a2* may be a target for EIC therapy.



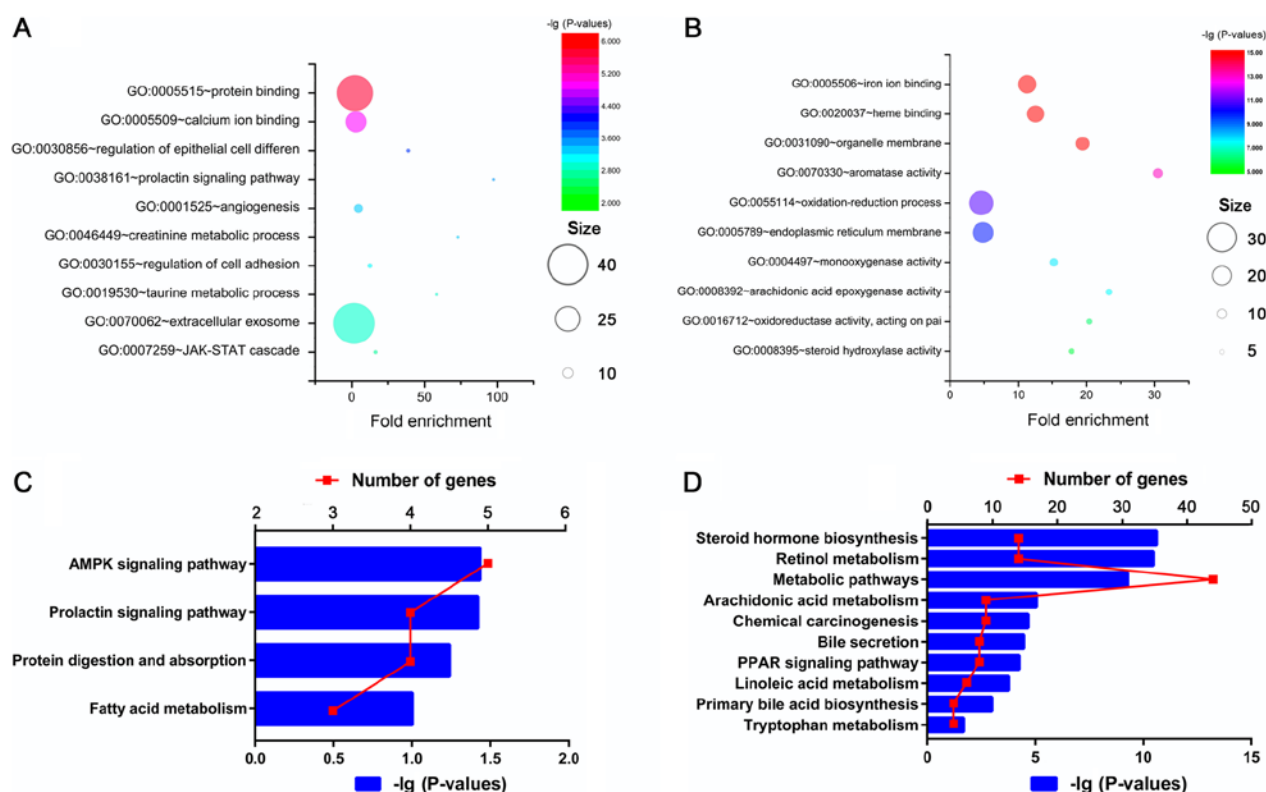


Figure 3. Functional enrichment analysis of DEGs. GO enrichment analysis of (A) upregulated DEGs and (B) downregulated DEGs. GO analysis classified into 3 groups, including 'Biological Process', 'Cellular Component' and 'Molecular Function'. KEGG enrichment analysis of (C) upregulated DEGs and (D) downregulated DEGs. DEGs, differentially expressed genes; GO, Gene ontology; KEGG, Kyoto Encyclopedia of Genes and Genomes; AMPK, 5'AMP-activated protein kinase.

Accumulation of hepatic bile acids often results in oxidative stress and inflammation, which then leads to hepatocellular injury (9). *Car3*, a human ortholog of *CA3*, is responsible for catalyzing the hydration of carbon dioxide in response to oxidative stress, but is nonetheless expressed at low levels in EIC. It has been demonstrated that *Car3* serves an important role in glutathionylation during cellular oxidative stress (35). Compared with parental cells, *Car3*-transfected cells exhibited lower levels of the intracellular reactive oxygen species (36). More importantly, *Car3* decreases the toxicity of hydrophobic bile acids on biliary epithelium in bicarbonate-rich hydrocholerisis (37). Additionally, *Car3* is involved in mediating oxidative stress in various tissues and pathological processes, including in skeletal muscle and during alcoholic liver disease and aging (38–40). As a nuclear-membrane enzyme, *Ltc4s* catalyzes the conjugation of leukotriene A4 to glutathione, generating leukotriene C4, which is the first reaction in the synthesis of cysteinyl leukotrienes (LTs) (41). The previously described role of LTs strongly implicates them in the pathogenesis of inflammatory diseases (41–42). In addition, increasing evidence has demonstrated that LTs are associated with cholestasis, hepatic inflammation during metabolic disease and fulminant hepatic failure (42). *Adam8* is a member of the ADAM protein family, which serves critical roles in certain liver diseases. Li *et al* (43), for example, demonstrated that *Adam8* promoted liver injury by inhibiting the proliferation of hepatocytes and promoting angiogenesis, and by affecting the metabolic function of the liver during acute liver injury induced by  $\text{CCl}_4$ . Accordingly, neutralization of *Adam8* ameliorated liver injury

and accelerated liver repair. Concomitantly, Higuchi *et al* (44) identified that oxazolone-induced contact hypersensitivity reactions were more severe in *Adam8* transgenic mice compared with wild-type mice. *Adam8*, directly or indirectly, regulates leukocyte infiltration in mice (44). The microarray data and RT-qPCR results from the present study indicated that *Car3*, *Ltc4s* and *Adam8* were the most significantly altered DEGs in the EE-induced rat model. Therefore, these 3 candidate genes may have crucial roles in the regulation of oxidative stress and inflammation during the pathogenesis of EIC.

Key pathways underlying EIC were investigated through KEGG and PPI network-associated module analyses in the present study. The KEGG analysis indicated that the AMPK signaling pathway was primarily enriched in the upregulated DEGs. It has previously been demonstrated that the activation of AMPK signaling is integral to the EE-mediated disruption of the expression of bile acid transporters and promoting the EIC process (45). In addition, inhibition of AMPK activation attenuated EE-induced cholestasis *in vitro* and *in vivo* (45). AMPK signaling is also crucial for the pathogenesis of cholestatic liver injury involved in regulating hepatic polarity, inflammation and fibrosis (46). Fatty acid metabolism was enriched in the upregulated DEGs, whereas steroid hormone biosynthesis and PPAR signaling pathway were enriched in the downregulated DEGs. These 3 pathways are all involved in lipid metabolism. It has been demonstrated previously that alterations in lipid metabolism may promote inflammation, fibrosis and proliferation in a mouse model of chronic cholestatic liver injury (47). Fenofibrate is a PPAR $\alpha$  agonist that protects against EE-induced

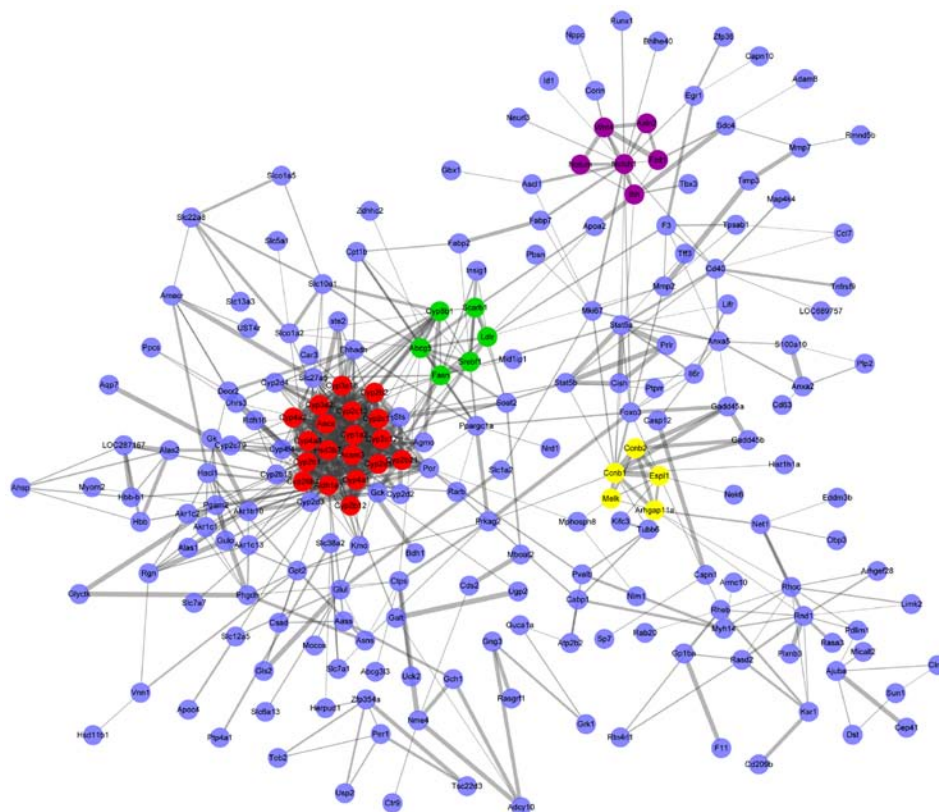


Figure 4. PPI network analysis of differentially expressed genes. The PPI network was constructed with the combined score of protein pairs  $>0.4$ . Line thickness indicates the strength of the association. The top 4 significant modules were selected according to the Molecular Complex Detection. The red dots represent module 1, the green dots represent module 2, the yellow dots represent module 3 and the purple dots represent module 4. PPI, protein-protein interaction.

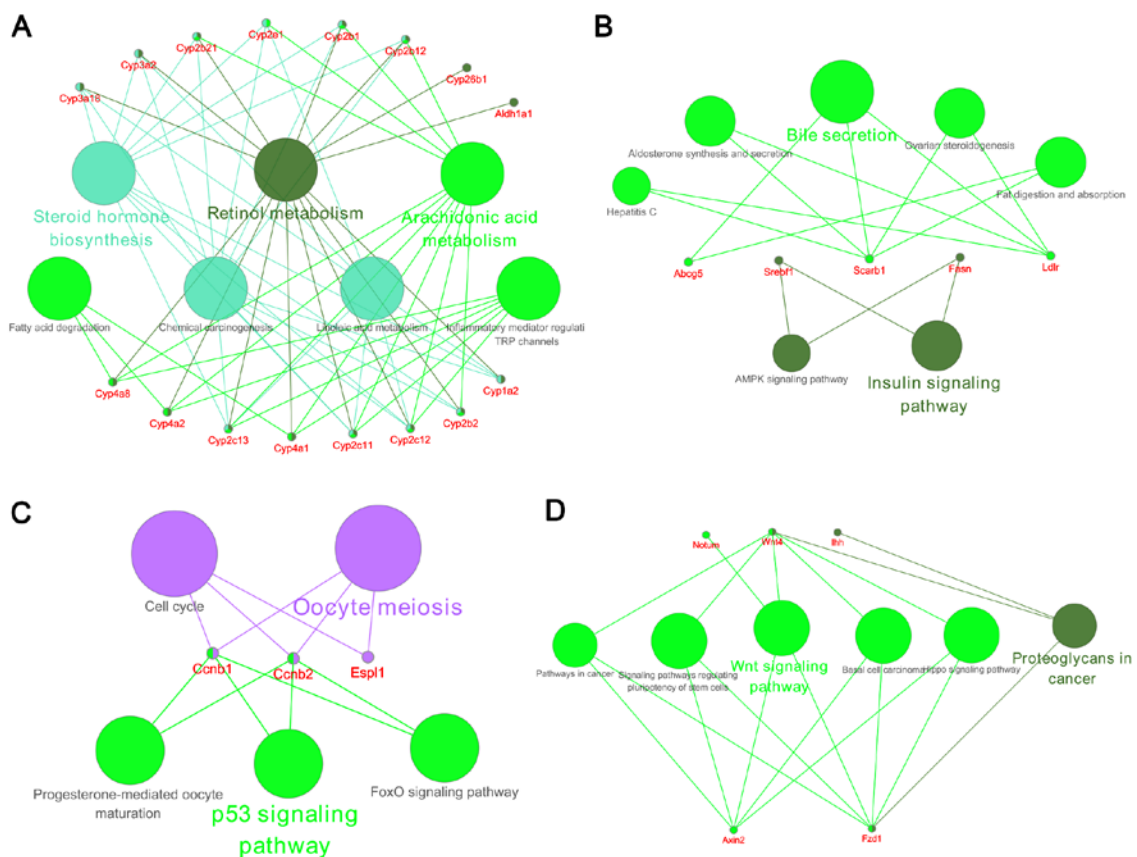


Figure 5. Pathway enrichment analysis of the top 4 modules from the protein-protein network by using ClueGO software. (A) Module 1. (B) Module 2. (C) Module 3. (D) Module 4.

cholestasis in mice (48). In addition, it is well recognized that bile acid accumulation contributes to cholestasis. The results of the present study indicated that bile secretion and primary bile acid biosynthesis were primarily enriched in the down-regulated DEGs, which suggests that bile acid homeostasis is disrupted during EE-induced cholestasis. In previous decades, numerous studies have struggled to regulate the expression of bile acid transporters, metabolic enzymes and nuclear receptors to improve bile acid homeostasis in cholestatic liver diseases (49). Accordingly, the inhibition of AMPK signaling or maintenance of lipid and bile acid homeostasis may provide potential strategies for EIC treatment.

The p53 and Wnt signaling pathways were identified as key pathways based on the data from the PPI network associated-modules. The role of p53 signaling on cholestasis is controversial. Chen *et al* (50) identified that the p53 activator doxorubicin attenuated cholic acid-induced cholestasis in mice through promoting the deposition of bile acid and alleviation of cholestatic syndrome, and that cholic acid-induced cholestatic liver injury was aggravated in p53-knockout mice. Concomitantly, other studies hypothesized that p53 signaling served an important role in promoting apoptosis during cholestatic liver injury (51,52). Future studies are required to determine the role of p53 signaling on EIC and other cholestatic diseases. In addition, it has been demonstrated that Wnt signaling is a critical regulator of the pathophysiology of cholestasis (53,54). Wnt signaling regulates hepatobiliary repair in cholestatic mice (54) and controls intrahepatic biliary network formation in zebrafish (53). Therefore, the regulation of p53 and Wnt signaling pathways may also provide novel insights into EIC treatment.

There are several limitations of the present study. Due to the fact that there are limited data available on the pathways and genes involved in EIC, the identified key pathways and candidate genes were not discussed in detail. Additionally, these results were generated from rat liver samples, which may cause tissue or sample heterogeneity in independent studies. Finally, only bioinformatics-based evidence and part of RT-qPCR data were presented, and additional studies are therefore required to confirm the functions of these candidate genes and key pathways in EIC, and to verify the results.

In summary, the present study identified 5 candidate genes and several key pathways that were demonstrated to be important in the pathogenesis of EIC. These pathways were involved in the homeostasis of lipids and bile acids and in AMPK, p53 and Wnt signaling. The results of the present study indicated that these candidate genes and key pathways may serve critical roles in the development and progression of EIC. Reversing the abnormal expression of candidate genes and dysfunction of key pathways may provide opportunities for novel EIC therapies.

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## Availability of data and materials

The microarray data and other data sets in the present study are available from the corresponding author on reasonable request.

## Authors' contributions

DL and CZ designed, supervised and revised the study. DX and CZ collected and analyzed the data, and wrote the manuscript. YX and WH contributed to microarray data collection and analysis. JY assisted in performing experiments and analysis of the data. All authors reviewed and approved the manuscript.

## Ethics approval and consent to participate

The present study was approved by the Ethical Committee on Animal Experimentation of Tongji Medical College, Huazhong University of Science and Technology (Wuhan, China).

## Patient consent for publication

Not applicable.

## Competing interests

The authors declare that they have no competing interests.

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