Screening and identification of key genes between liver hepatocellular carcinoma (LIHC) and cholangiocarcinoma (CHOL) by bioinformatic analysis

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Abstract

Background: Liver hepatocellular carcinoma (LIHC) and cholangiocarcinoma (CHOL) are common primary liver cancers worldwide. Liver stem cells have biopotential to differentiate into either hepatocytes and cholangiocytes, the phenotypic overlap between LIHC and CHOL has been acceptable as a continuous liver cancer spectrum. However, few studies directly investigated the underlying molecular mechanisms between LIHC and CHOL.

Method: To identify the candidate genes between LIHC and CHOL, three data series including GSE31370, GSE15765 and GSE40367 were downloaded from Gene Expression Omnibus (GEO) database. The differentially expressed genes (DEGs) were identified, and function enrichment analyses were performed. The protein-protein interaction network (PPI) was constructed and the module analysis was performed using STRING and Cytoscape.

Results: A total of 171 DEGs were identified, consisting of 49 downregulated genes and 122 upregulated genes. Compared with CHOL, the enriched functions of the DEGs mainly included steroid metabolic process, acute inflammatory response, coagulation. Meanwhile, the pathway of KEGG enrichment analyses showed that the upregulated gene(s) were mainly enriched complement and coagulation cascades, cholesterol metabolism and PPAR signaling pathway, while the downregulated gene(s) were mainly enriched in ECM-receptor interaction, focal adhesion, bile secretion. Similarly, the most significant module was identified and biological process analysis revealed that these genes were mainly enriched in regulation of blood coagulation, acute inflammatory response, complement and coagulation cascades. Finally, two (ITIH2 and APOA2) of 10 hub genes had been screened out to help differential diagnosis.

Conclusion: 171 DEGs and two (ITIH2 and APOA2) of 10 hub genes identified in the present study help us understand the different molecular mechanisms between LIHC and CHOL, and provide candidate targets for differential diagnosis.

Abbreviations: APOA1 = apolipoprotein A1, APOA2 = apolipoprotein A2, BP = biological process, CC = cellular component, CHOL = cholangiocarcinoma, DEGs = differentially expressed genes, GEO = Gene Expression Omnibus, GO = Gene Ontology, GTEx = Genotype-Tissue Expression, HA = hyaluronic acid, ITI = inter-alpha-trypsin inhibitors, KEGG = Kyoto Encyclopedia of Genes and Genomes, LIHC = liver hepatocellular carcinoma, MCODE = Molecular Complex Detection, MF = molecular function, PPI = protein-protein interaction network, TCGA = the Cancer Genome Atlas.

Keywords: cholangiocarcinoma (CHOL), differentially expressed genes (DEGs), gene expression omnibus (GEO), liver hepatocellular carcinoma (LIHC)

1. Introduction

The major primary liver cancers in adults consists of 3 main pathological patterns: liver hepatocellular carcinoma (LIHC), cholangiocarcinoma (CHOL), and combined hepatocellular-cholangiocarcinoma (CHC). As the second most common primary liver, CHOL is widely considered as a heterogeneous group of cancers with pathologic features of biliary tract differentiation.[1] But there are some different viewpoints from...
other literatures, which indicated CHOL originated in transdifferentiation of hepatocytes.\textsuperscript{[2,3]} Because liver stem cells have biopotential to differentiate into either hepatocytes or cholangiocytes, the phenotypic overlap between LIHC and CHOL has been acceptable as a continuous liver cancer spectrum.\textsuperscript{[4–6]} Consistent with this opinion, the study of Farshidfar et al indicated LIHC and CHOL lie along a spectrum of primary liver carcinomas, through analysis of 600 genes that are most enriched in these tumors.\textsuperscript{[6]} However, there were etiologic, biological heterogeneity, therapeutic plan, even survival and prognosis differences between them.\textsuperscript{[9]} When it comes to etiology, risk factors like viral hepatitis and alcoholic as we as non-alcoholic steatohepatitis are much stronger for LIHC, while history of gallstones, liver fluke infestation, and primary sclerotic cholangitis are associated with higher risk of CHOL.\textsuperscript{[7]} The distinction between them is important clinically as treatment considerations differ for LIHC and CHOL. In early stage, surgery, liver transplantation, and localized ablative techniques can be regarded as radical treatments for LIHC. On the other hand, CHOL is not a widely accepted indication for liver transplantation.\textsuperscript{[10]} In advanced LIHC, the multi-kinase inhibitors sorafenib, levatinib, and combination immunotherapy are standard first-line therapy options.\textsuperscript{[9–11]} while unresectable CHOL are typically treated with gemcitabine combined with cisplatin therapy.\textsuperscript{[12]} Although a significant amount of time and efforts have been taken on understanding primary liver cancer, there is still an incomplete appreciation of the exact mechanisms between LIHC and CHOL requiring urgent consideration.

Dynamic CT scanning and magnetic resonance imaging can help distinguish between LIHC and CHOL. However, some small mass-forming CHOL may mimic hepatocellular carcinoma, leading lack of specificity. Differential diagnosis of LIHC and CHOL represents a current clinical challenge. With the advancement of gene chips and high-throughput second-generation sequencing technologies widely used for understanding gene functions and biological patterns, identifying biomarkers for disease classification and diagnosis, increasingly more genetic data in public databases is stored to mine.\textsuperscript{[13]} Microarray technology may help us to understanding the molecular basis between LIHC and CHOL. In this study, we aimed to identify DEGs between LIHC and CHOL from the Gene Expression Omnibus (GEO) database (https://www.ncbi.nlm.nih.gov/geo/). Subsequently, Gene Ontology (GO) terminology and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway enrichment analysis, and protein–protein interaction (PPI) analysis were performed to screen for key genes and biological pathways. Finally, analysis and compare the level of hub gene expression level between LIHC/CHOL samples and normal samples. These analysis results can provide new perspective about their cellular origins and differential diagnosis.

### 2. Material and methods

#### 2.1. Data source

GEO database is a public functional genomics data repository of high throughout gene expression data, chip, and microarrays. We obtained gene expression profile data from the GEO database based on the keywords “hepatocellular carcinoma”, “cholangiocarcinoma”, and “homo sapiens”. Three GEO series (GSE31370, GSE15765, GSE40367) were retrieved. GSE31370 was retrieved from platform GPL571 (Illumina Human Genome U133A 2.0 Array) and GPL570 ([HG-U133_Plus_2] Affymetrix Human Genome U133A 2.0 Array) respectively. First, we excluded mixed hepatocellular cell carcinoma like combined hepatocellular-cholangiocarcinoma, because it has intermediate characteristics between experimental group including LIHC samples and control group including CHOL samples. Subsequently, all tissue samples came from primary tumor (liver) not metastasis tumor (lung, adrenal gland, and lymph node) to reduce the potential influence of spatial heterogeneity. Finally, we included the GSE31370 dataset contained 6 CHOL samples and 15 LIHC samples. The GSE15765 contained 13 CHOL samples and 70 LIHC samples. The GSE40367 contained 4 CHOL samples and 15 LIHC samples (Table 1). Ethical approval was waived since this study used only publicly available data, and did not involve any experiment on humans.

#### 2.2. Data processing and DEGs analysis

The DEGs of GSE31370, GSE15765 and GSE40367 were obtained by GEO2R online tool (https://www.ncbi.nlm.nih.gov/geo/geo2r).\textsuperscript{[14]} Screening of DEGs between iCCA and HCC as well as volcano mapping were performed using SangerBox. And the gene met the statistically significant criteria of | log FC (fold change) | \geq 1.0 and P value <.05 were considered the presence of DEGs. Then, The Venn diagram web tool (http://bioinformatics.psb.ugent.be/webtools/Venn/) was performed to identify the common DEGs shared among these 3 GSEs.

#### 2.3. GO function and KEGG pathway enrichment analyses of DEGs

To analyze the function of these DEGs, using a biological analysis tool called DAVID online database (version 6.7) (http://david.ncifcrf.gov) provides a comprehensive set of functional annotation information of genes. GO is a major bioinformatics tool to annotate genes and analyze biological functions, which consists of 3 parts including biological process (BP), cellular component (CC), and molecular function (MF). After that, Enrichr database (http://amp.pharm.mssm.edu/Enrichr) was used to perform

### Table 1

Statistics of 3 data series derived from GEO database.

| GEO series | CHOL | LIHC | Total | Tissue source |
|------------|------|------|-------|---------------|
| GSE31370   | 6    | 15   | 26    | liver         |
| GSE15765   | 13   | 70   | 90    | liver         |
| GSE40367   | 4    | 15   | 61    | liver         |

CHOL = cholangiocarcinoma, GEO = Gene Expression Omnibus, GSE = GEO series, LIHC = liver hepatocellular carcinoma.
KEGG pathway enrichment analysis between the LIHC group and CHOL group. KEGG is a database resource for understanding high-level functions and biological systems from large-scale molecular datasets generated by high-throughput experimental technologies.[15] P < .05 was considered statistically significant.

2.4. PPI network construction and module analysis

The PPI network among the common DEGs was predicted by Search Tool for the Retrieval of Interacting Genes (STRING; http://string-db.org/) (version 11.0) online database. Analyzing the functional interactions between proteins may provide insights into the mechanisms of generation or development of diseases. The PPI networks were constructed using Cytoscape software (version 3.8.0) and the most significant module in the PPI networks was identified using a plugin called Molecular Complex Detection (MCODE) (version 1.6.1). We set the criteria for selection as follows: MCODE scores > 5, degree cutoff = 2, node score cutoff = 0.2, Max depth = 100 and k-score = 2.

2.5. Hub genes selection and analysis of hub genes

The top 10 nodes with highest degree of PPI network connectivity were identified as hub genes, using CytoHubba plugin in Cytoscape. We use GEPIA as a web server for analyzing the RNA sequencing expression data from the Cancer Genome Atlas (TCGA) and the Genotype-Tissue Expression (GTEx) projects to compare these identified the top 10 hub genes between LIHC/CHOL with normal samples including differential expression analysis and patient survival analysis.[16]

3. Results

3.1. Identification of common DEGs across 3 GSEs between LIHC and CHOL

In our study, 3 data series were selected from GEO database (Table 1). According to the selection criteria of DGEs, these DGEs (381 in GSE31370, 3137 in GSE15765 and 3846 in GSE40367) were identified (Fig. 1). The overlap among the 3 data series contained 171 DEGs as shown in the Venn diagram, containing 123 upregulated genes and 48 downregulated genes (Fig. 2A).

3.2. GO term and KEGG pathway enrichment analyses of the common DEGs

Functional and pathway enrichment analyses were performed using DAVID and Enrichr. GO analysis results showed that changes in BP of DEGs were mainly enriched in steroid metabolic process, response to wounding, cholester metabolism process, acute inflammatory response, regulation of response to external stimulus, wound healing, blood coagulation (Table 2). Changes in MF were significantly enriched in endopeptidase inhibitor activity serine-type endopeptidase inhibitor activity, peptidase inhibitor activity, enzyme inhibitor activity (Table 2). Changes in CC of DEGs were mainly enriched in extracellular space, integral and intrinsic to plasma membrane (Table 2). KEGG pathway analysis showed that the upregulated DEGs were mainly enriched in complement and coagulation cascades, cholesterol metabolism, and PPAR signaling pathway, while the downregulated DEGs enriched in ECM-receptor interaction, focal adhesion, and bile secretion (Fig. 3A and 3B).

3.3. PPI network construction and module analysis

The PPI network of DEGs and the most significant module were predicted by STRING and constructed by Cytoscape (Fig. 2B and Fig. 2C). In the most significant module from PPI network, there are 30 nodes and 369 edges. Functional analysis results of genes in this module indicated that this module was enriched in complement and coagulation cascades, cholesterol metabolism, and PPAR signaling pathway as well as the downregulated DEGs enriched in ECM-receptor interaction, focal adhesion, and bile secretion (Fig. 3C and Table 3).

3.4. Hub genes and analysis of hub genes

Using CytoHubba identified hub genes, which satisfied top 10 of PPI network connectivity (Fig. 4). The names and abbreviations of the top 10 genes are shown in Table 4. Subsequently, we use GEPIA to compare the expression of the top 10 hub genes between cholangiocarcinoma / liver hepatocellular carcinoma with normal samples (Fig. 5). Finally, the prognostic value of APOA2 and ITIH2 were evaluated by Kaplan–Meier analysis using GEPIA (Fig. 6 and Fig. 7).
4. Discussion

Previous studies considered that liver stem cells have bipotential capacity to differentiate into hepatocytes and cholangiocytes. Additionally, the existence of combined hepatocellular-cholangiocarcinoma cases also reinforce liver progenitor cell might be the origin both entities. Therefore, there is some truth to the view that LIHC and CHOL showed partial homology in the tumorigenesis and progression. Microarray technology may help us to understanding the substantial differences in the molecular mechanisms between LIHC and CHOL.

In this study, 3 mRNA microarray datasets of LIHC tissues and CHOL tissues were analyzed to obtain common potential DGEs, containing 123 upregulated genes and 48 downregulated genes. Compared with CHOL, the BP of GO enrichment analyses of LIHC were mainly enriched in steroid metabolic process.
response to wounding, acute inflammatory response and coagulation (Table 2). Meanwhile, the pathway of KEGG enrichment analyses showed that the upregulated gene were mainly enriched complement and coagulation cascades, cholesterol metabolism and PPAR signaling pathway, while the downregulated gene were mainly enriched in ECM-receptor interaction, focal adhesion, bile secretion (Fig. 3). Previous studies have indicated complement activation can be regard as a tumor-promoting factor.[17] Moreover, PPAR signaling pathway regulates the expression of various including fatty acid degradation, glycerophospholipid metabolism, which often involved in carcinogenesis, progression, and metabolic state of LIHC.[18,19] The results are consistent with experimental results reported by Chen, whose hypothesis indicated that the aberrant lipid metabolic pathway play a significant role in LIHC, while high serum level of bile acids founded in patients with CHOL.[20-22]

From clinicopathological, steatosis as a common finding was detectable in LIHC, while CHOL differentiation is characterized by mucin-producing biliary epithelium forming true glandular structures and surrounding desmoplastic stroma.[18,23] Additionally, none of the LIHC samples showed intracellular mucin, whereas all of the CHOL showed mucin formation.[24] Extracellular matrix (ECM) is composed of several macro-molecules associated in a complex network. Hyaluronic acid (HA) is a major representative of ECM. ECM-receptor interactions lead to a direct or indirect control of cellular activities, which is associated with adhesion, migration, and differentiation.[25] The KEGG enrichment analyses of LIHC indicated significant down-regulation of ECM-receptor interaction and focal adhesion, suggesting less aggressive phenotype than CHOL to some extent. Our findings is consistent with the previous findings that CHOL showed poorer clinical outcome than LIHC.[24,26]

Similarity, through the most significant module, GO enrichment analysis of these up-regulation genes were mainly enriched in response to wounding, acute inflammatory response, and coagulation, while changes in KEGG were mainly enriched in complement and coagulation cascades, compared to that of CHOL. The results may have possible related with etiology. Risk factors for LIHC are condition of continuous liver injury and chronic inflammation. In the PPI network constructed by STRING and Cytoscape, the upregulated top 10 hub genes of distinction between LIHC and CHOL were identified, including FGA, FGG, ITIH2, AHSG, APOA1, APOB, F2, APOA2, PROC, KNG1. However, only the expression of ITIH2 and APOA2 were able to distinguish between LIHC and CHOL significantly (Fig. 5).

ITIH2, as inter-alpha-trypsin inhibitor heavy chain 2, is one of heavy chain in the inter-alpha-trypsin inhibitors (ITI), which are a family of plasma protease inhibitors. The family of heavy chains contains 5 genes: ITIH1, ITIH2, ITIH3, ITIH4, and ITIH5.[27] Of these, ITIH2 and ITIH5 are arranged on chromosome 10p15.[28,27] In vitro experiment, ITIH1 and ITIH3 over-expression increased cell attachment and induced decrease of metastasis number.[28] Similarly, Himmelfarb founded that normal breast epithelial cells clearly express ITIH5, while the expression of ITIH5 is consistently lost or strongly down-regulated in invasive breast cancer.[29] Moreover, patients with abundant ITIH5 expression had a better clinical outcome, compared to those with reduced expression in invasive node-negative breast cancer.[30] These results clearly indicated that the ITI family may contribute to carcinogenesis via deregulated gene expression. Because ITIH2 and ITIH5 are located on chromosome 10p15, their strong correlation indicated that these molecules interact in their tumor-suppressive and metastasis-repressive properties. The overall survival analysis of LIHC patients with respect to expression status of ITIH2 is relatively consistent with the previous study (Fig. 7D).

| Category | Term | Pathway description | Count in gene set | Adjusted P |
|----------|------|---------------------|------------------|-----------|
| Upregulated | BP term | GO:00068202 | steroid metabolic process | 19 | 4.58E-11 |
| | BP term | GO:00069611 | response to wounding | 25 | 2.68E-09 |
| | BP term | GO:0016125 | sterol metabolic process | 13 | 1.25E-08 |
| | BP term | GO:0008203 | cholesterol metabolic process | 12 | 6.16E-08 |
| | BP term | GO:0025266 | acute inflammatory response | 12 | 9.87E-08 |
| | BP term | GO:0002101 | regulation of response to external stimulus | 14 | 1.04E-07 |
| | BP term | GO:0042060 | wound healing | 14 | 8.55E-07 |
| | BP term | GO:0007596 | blood coagulation | 11 | 1.55E-06 |
| | BP term | GO:0008817 | coagulation | 11 | 1.55E-06 |
| | BP term | GO:00030193 | regulation of blood coagulation | 8 | 1.97E-06 |
| | CC term | GO:0005615 | extracellular space | 33 | 1.17E-13 |
| | CC term | GO:0005576 | extracellular region | 50 | 2.31E-11 |
| | CC term | GO:0044421 | extracellular region part | 33 | 3.88E-10 |
| | MF term | GO:0004866 | endopeptidase inhibitor activity | 13 | 8.73E-07 |
| | MF term | GO:0004867 | serine-type endopeptidase inhibitor activity | 11 | 7.23E-07 |
| | MF term | GO:0003014 | peptidase inhibitor activity | 13 | 5.38E-07 |
| | MF term | GO:0004857 | enzyme inhibitor activity | 16 | 4.87E-07 |
| Downregulated | CC term | GO:0005887 | integral to plasma membrane | 12 | 0.034720371 |
| | CC term | GO:0001226 | intrinsic to plasma membrane | 12 | 0.035406774 |

BP = biological process, CC = cellular component, DEGs = differentially expressed genes, GO = Gene Ontology, MF = molecular function.
seen in 70% of breast cancers, 71% of lung cancers, and 70% of renal tumors. The lower expression of ITIH2 in CHOL patients concur with these of several previous studies (Fig. 5C). As for the aspects of ITIH2 expression in LIHC, patients with highly expression of ITIH2 would have a better overall survival ($P = .019$). However, the findings of ITIH2 expression in LIHC patients is higher than that of normal group, which seem to conflict with those of previous research. It is probably worth to do further research on this subject.

Apolipoprotein A2 (APOA2), encoded by the APOA2 gene, is the second-most major apolipoprotein of the high-density lipoprotein cholesterol. APOA2 is part of the apolipoprotein
superfamily including apolipoprotein A, apolipoprotein C and apolipoprotein E families. Studies supported APOA2 has been associated with lipid-related diseases through modulating triglyceride, cholesterol transport, and glucose metabolism. Previously published clinical studies suggested that APOA2 has been associated with various types of cancer. APOA2 levels are decreased in pancreatic and metastatic renal cell cancer. Plasma APOA2 could improve an efficiency of detection of pancreatic cancer. However, the elevated level of APOA2 and APOA1 urinary protein play a significant role in early detection of urinary bladder cancer. In our study, APOA2 was significantly decreased in CHOL subgroups, whereas it was significantly increased in LIHC subgroups (Fig. 5H). When it comes to the prognosis, no significant correlation was detected between APOA2 and prognosis of LIHC and CHOL.

We were not able to demonstrate a statistically significant association between APOA2 expression in LIHC and reduced overall survival or shorter disease-free survival in Kaplan–Meier analysis, and neither did CHOL (Fig. 6 and Fig. 7). However, there was statistically significant correlation between lower expression of ITIH2 in LIHC and reduced overall survival.

### 5. Conclusion

By using bioinformatic analysis, the present study was designed to identify DEGs that may be distinguish LIHC and CHOL. A total of 171 DEGs and 10 hub genes may be regarded as biomarkers. Especially, ITIH2 and APOA2 were 2 potential key genes, which may induce to differentiate into LIHC rather than CHOL. Furthermore, the higher expression status of ITIH2 in LIHC patients is associated with favorable overall survival. Our results may provide new insights of molecular mechanisms regarding LIHC and CHOL, however, further experimental studies are still need to elucidate the biological function.

### Table 3

| Category | Term | Pathway description | Count in gene set | Adjusted P |
|----------|------|---------------------|------------------|-----------|
| BP term  | GO:0009611 | response to wounding | 16 | 1.06E-11 |
| BP term  | GO:0030193 | regulation of blood coagulation | 8 | 3.64E-10 |
| BP term  | GO:0050818 | regulation of coagulation | 8 | 3.43E-09 |
| BP term  | GO:0032101 | regulation of response to external stimulus | 10 | 1.43E-08 |
| BP term  | GO:0042060 | wound healing | 10 | 1.91E-08 |
| BP term  | GO:0006953 | acute-phase response | 7 | 8.61E-08 |
| BP term  | GO:0030195 | negative regulation of blood coagulation | 6 | 7.76E-08 |
| BP term  | GO:0025289 | acute inflammatory response | 8 | 9.16E-08 |
| BP term  | GO:0050817 | coagulation | 8 | 9.16E-08 |
| BP term  | GO:007599 | blood coagulation | 8 | 1.17E-07 |
| BP term  | GO:0007599 | hemostasis | 8 | 1.12E-07 |
| CC term  | GO:0005615 | extracellular space | 29 | 5.54E-20 |
| CC term  | GO:0005576 | extracellular region | 22 | 1.77E-19 |
| CC term  | GO:0044421 | extracellular region part | 22 | 1.26E-16 |
| MF term  | GO:0004857 | enzyme inhibitor activity | 14 | 3.38E-13 |
| MF term  | GO:004866 | endopeptidase inhibitor activity | 12 | 2.47E-13 |
| MF term  | GO:0030414 | peptidase inhibitor activity | 12 | 2.99E-13 |
| KEGG pathway | hsa04610 | Complement and coagulation cascades | 10 | 4.97E-14 |

BP = biological process, CC = cellular component, DEGs = differentially expressed genes, GO = Gene Ontology, KEGG = Kyoto Encyclopedia of Genes and Genomes, MF = molecular function.

### Table 4

| Gene symbol | Gen description            | Degree |
|-------------|----------------------------|--------|
| FGA         | fibrinogen alpha chain     | 29     |
| FGG         | fibrinogen gamma chain     | 29     |
| ITIH2       | inter-alpha-trypsin inhibitor heavy chain | 29 |
| AHSG        | alpha 2-HS glycoprotein    | 29     |
| APOA1       | apolipoprotein A1          | 28     |
| APOB        | apolipoprotein B            | 28     |
| F2          | coagulation factor II       | 28     |
| APOA2       | apolipoprotein A2           | 27     |
| PROC        | protein C                  | 27     |
| KNG1        | kininogen 1                | 27     |

Figure 4. Top 10 hub genes of connectivity in the PPI network.
Figure 5. Expression analysis of top 10 hub genes between LIHC/CHOL and corresponding normal tissues by GEPIA. (A) FGA expression between CHOL/LIHC and normal tissues; (B) FGG expression between CHOL/LIHC and normal tissues; (C) ITIH2 expression between CHOL/LIHC and normal tissues; (D) AHSG expression between CHOL/LIHC and normal tissues; (E) APOA1 expression between CHOL/LIHC and normal tissues; (F) APOB expression between CHOL/LIHC and normal tissues; (G) F2 expression between CHOL/LIHC and normal tissues; (H) APOA2 expression between CHOL/LIHC and normal tissues; (I) PROC expression between CHOL/LIHC and normal tissues; (J) KNG1 expression between CHOL/LIHC and normal tissues. (∗P < .05).
Figure 6. Disease-free survival analysis of CHOL patients and LIHC patients with respect to APOA2 and ITIH2 expression status.
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