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WJGO mainly publishes articles reporting research results and findings obtained in the field of gastrointestinal oncology and covering a wide range of topics including liver cell adenoma, gastric neoplasms, appendiceal neoplasms, biliary tract neoplasms, hepatocellular carcinoma, pancreatic carcinoma, cecal neoplasms, colonic neoplasms, colorectal neoplasms, duodenal neoplasms, esophageal neoplasms, gallbladder neoplasms, *etc.*

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Differences of core genes in liver fibrosis and hepatocellular carcinoma: Evidence from integrated bioinformatics and immunohistochemical analysis

Yue Li, Shou-Li Yuan, Jing-Ya Yin, Kun Yang, Xin-Gang Zhou, Wen Xie, Qi Wang

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Abstract

BACKGROUND

Liver fibrosis and hepatocellular carcinoma (HCC) are common adverse consequences of chronic liver injury. The interaction of various risk factors may cause them to happen. Identification of specific biomarkers is of great significance for understanding the occurrence, development mechanisms, and determining the novel tools for diagnosis and treatment of both liver fibrosis and HCC.

AIM

To identify liver fibrosis-related core genes, we analyzed the differential expression pattern of core genes in liver fibrosis and HCC.

METHODS

Gene expression profiles of three datasets, GSE14323, GSE36411, and GSE89377, obtained from the Gene Expression Omnibus (GEO) database, were analyzed, and differentially expressed genes (DEGs) between patients with liver cirrhosis and healthy controls were identified by screening *via* R software packages and online tool for Venn diagrams. The WebGestalt online tool was used to identify DEGs enriched in biological processes, molecular functions, cellular components, and Kyoto Encyclopedia of Genes and Genomes pathways. The protein-protein interactions of DEGs were visualized using Cytoscape with STRING. Next, the

expression pattern of core genes was analyzed using Western blot and immunohistochemistry in a carbon tetrachloride (CCl₄)-induced liver cirrhosis mouse model and in patient liver samples. Finally, Kaplan-Meier curves were constructed using the Kaplan-Meier plotter online server.

RESULTS

Forty-five DEGs (43 upregulated and 2 downregulated genes) associated with liver cirrhosis were identified from three GEO datasets. Ten hub genes were identified, which were upregulated in liver cirrhosis. Western blot and immunohistochemical analyses of the three core genes, decorin (DCN), dermatopontin (DPT), and SRY-box transcription factor 9 (SOX9), revealed that they were highly expressed in the CCl₄-induced liver cirrhosis mouse model. The expression levels of DCN and SOX 9 were positively correlated with the degree of fibrosis, and SOX 9 level in HCC patients was significantly higher than that in fibrosis patients. However, high expression of DPT was observed only in patients with liver fibrosis, and its expression in HCC was low. The gene expression profiling interactive analysis server (GEPIA) showed that SOX9 was significantly upregulated whereas DCN and DPT were significantly downregulated in patients with HCC. In addition, the Kaplan-Meier curves showed that HCC patients with higher SOX9 expression had significantly lower 5-year survival rate, while patients with higher expression of DCN or DPT had significantly higher 5-year survival rates.

CONCLUSION

The expression levels of DCN, DPT, and SOX9 were positively correlated with the degree of liver fibrosis but showed different correlations with the 5-year survival rates of HCC patients.

Key Words: Liver cirrhosis; Hepatocellular carcinoma; Bioinformatical analysis; Decorin; Dermatopontin; SRY-box transcription factor 9

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Core Tip: GSE14323, GSE36411, and GSE89377 are available from the Gene Expression Omnibus database. Forty-five differentially expressed genes and 10 hub genes were identified between cirrhotic and healthy livers. quantitative polymerase chain reaction, Western blot, and immunohistochemical analyses showed that decorin (DCN), dermatopontin (DPT), and SRY-box transcription factor 9 (SOX9) were highly expressed in the CCl₄-induced cirrhotic mouse model. The expression level of SOX9 was also significantly increased in HCC patients, and was associated with the fibrosis stage. However, overexpression of DPT was only observed in patients with liver fibrosis. The Kaplan-Meier curves showed that HCC patients with higher SOX9 expression had significantly lower 5-year survival rate, while patients with higher expression of DCN or DPT had higher 5-year survival rates.

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INTRODUCTION

Chronic liver infection, including chronic hepatitis B (CHB), chronic hepatitis C, alcoholic liver disease, and non-alcoholic fatty liver disease can all result in liver fibrosis. Primary liver cancer is the seventh most common cancer worldwide[1]. Hepatocellular carcinoma (HCC), which is the dominant type of liver cancer, accounts for approximately 75% of all liver cancers worldwide[2] and is the second most fatal disease in China[3]. Liver cirrhosis is an advanced stage of liver fibrosis and is characterized by limited regeneration capacity and serious complications[4]. Most HCCs develop in the background of chronic liver injury, hepatic inflammation, and liver fibrosis. Unfortunately, to date, there are still no effective treatment strategies for liver cirrhosis, and the limited number of specific biomarkers for HCC related to fibrosis further compounds the problem of its diagnosis and treatment[5,6].

The pathogenesis of liver fibrosis and HCC is complex as the interaction of many factors may lead to their occurrence. In recent years, with the optimization of gene sequencing platforms, several differentially expressed genes (DEGs) have been identified using bioinformatics analysis[7,8]. To date, there is a huge collection of data stored in the Gene Expression Omnibus (GEO) database of gene expression that

can be explored to find the relevant DEGs for a diseased condition. Chan *et al*[9] identified DEGs between cirrhotic and non-cirrhotic livers using microarray gene analysis. Many human genes may show differential expression patterns and functions with the onset of fibrosis and/or HCC. However, the study by Chan *et al*[9] was limited by the small sample size, which only included 24 patients with cirrhosis and 16 patients without cirrhosis. The results obtained solely from either bioinformatics or experimental approach may not elucidate relevant DEGs. Hence, integrating bioinformatics methods with experimental techniques may help us to better understand the underlying mechanisms behind fibrosis/HCC pathogenesis.

In this study, we analyzed three databases from GEO, R software packages and online tools to identify DEGs, including upregulated and downregulated genes between liver fibrosis and HCC. The molecular function, cellular component, biological process, and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathways of DEGs were then assessed. We also constructed a protein-protein interaction (PPI) network using Cytoscape for further analysis. Using these methods, hub genes were identified and subjected to KEGG pathway enrichment analysis. Finally, real-time quantitative polymerase chain reaction (qPCR), Western blotting, and immunohistochemistry of the liver tissue samples from mouse model and patients were carried out to identify novel biomarkers of liver fibrosis. Our study identified fibrosis-related core genes and compared their phenotypic differences between liver fibrosis and HCC.

MATERIALS AND METHODS

Patient liver samples collection

Liver tissue samples were collected from 5 healthy controls, 40 patients with CHB ($n = 28$) and CHB-associated HCC ($n = 12$) at the Beijing Ditan Hospital, Capital Medical University, Beijing, China. The diagnosis of CHB was based on the "Guidelines on prevention and treatment of chronic hepatitis B in China"[10]. Chronic HBV infection is defined as the persistence of HBsAg in blood serum for at least 6 mo. Patients who were diagnosed for Hepatitis C viral infection, drug-induced liver disease, non-alcoholic liver disease, alcoholic liver disease, autoimmune liver disease, cholestatic liver disease, or hereditary metabolic liver disease were excluded. All samples were analyzed by a clinician and two independent pathologists with no prior knowledge of demographic and clinical data. The degree of liver inflammation and fibrosis were scored according to the METAVIR system[11], and liver samples were divided into five groups: normal control, fibrosis grade 0 (S0), fibrosis grade 1-2 (S1-2), fibrosis grade 3-4 (S3-4), and HCC group. Meanwhile, we collected the clinical data, including sex, age, HBeAg, HBV DNA, alanine transaminase, aspartate transaminase, total bilirubin, albumin, cholinesterase and alpha-fetoprotein. The study protocol conformed to the ethical guidelines of the 1975 Declaration of Helsinki, as approved by the Ethics Committee of Beijing Ditan Hospital.

Animals

Six-week-old male C57BL/6 mice were purchased from Vital River Laboratory Animal Technology Co., Ltd, Beijing, China. All mice were housed in a specific pathogen free laboratory animal house (Institute of Zoology of Beijing, Chinese Academy of Sciences, China) at 24 °C with a 12 h light/dark cycle. All animal studies were approved by the Institutional Animal Care and Use Committee of the Institute of Zoology, Chinese Academy of Sciences.

Twelve male C57BL/6 mice were randomly divided into two groups: control group and carbon tetrachloride (CCl₄)-treated group. To induce liver cirrhosis, CCl₄ (0.5 µL/g) mixed with corn oil was intraperitoneally injected into the mice three times per week for 12 wk. The control group was injected with an equal volume of corn oil. CCl₄ was purchased from Sigma-Aldrich (St. Louis, MO, United States).

Histological and immunohistochemical analysis

For hematoxylin and eosin (HE) staining, Masson's trichrome staining, and immunohistochemical analysis, liver tissues collected from mouse model or patients were fixed with 4% paraformaldehyde solution and embedded in paraffin. For histological analysis, 5 µm thick sections were stained and observed under 10 × or 20 × objective lens. Masson's trichrome kit (G1281, Solarbio, Beijing, China) was used according to the manufacturer's instructions.

For immunohistochemical staining, 5% bovine serum albumin in 0.1% TritonX-100 tris-buffered saline was used as the blocking solution. The samples were incubated overnight at 4 °C with anti-decorin (DCN) (ab277636, Abcam), anti-dermatopontin (DPT) (10537-1-AP, Proteintech), and anti-SRY-box transcription factor 9 (SOX9) (ab185966; Abcam) antibodies. After incubation with a peroxidase-conjugated secondary antibody, the signal was visualized using a diaminobenzidine peroxidase substrate kit. The collagen area or positive area of immunohistochemical staining was quantified using ImageJ 1.52a software.

Real-time qPCR

Total RNA from liver tissues was isolated using TRIzol™ reagent (Thermo Fisher Scientific, MA, United States). Isolated RNA was reverse-transcribed into complementary DNA (cDNA) using a high-capacity cDNA reverse transcription kit (Promega, WI, United States). The relative expression of genes was detected by real-time fluorescence qPCR system (Light Cycler 480, Roche, Sweden) with SYBR green master mix (Promega, WI, United States). The primer sequences used in this study were listed in [Supplementary Table 1](#). Statistical significance between the control and CCl₄-treated groups was defined at $P < 0.05$.

Western blotting

Total protein from liver tissues was extracted using radioimmunoprecipitation assay buffer. The protein concentration of the samples was measured by bicinchoninic acid assay. The same concentration of protein was loaded to 10% sodium dodecyl sulfate polyacrylamide gel and then transferred to a polyvinylidene fluoride membrane. The membranes were incubated overnight at 4°C in anti- α -SMA (ab5694, Abcam), anti-DCN (ab277636, Abcam), anti-DPT (10537-1-AP, Proteintech), and anti-SOX9 (ab185966, Abcam) antibodies. All signals were visualized by density scanning (Image Quant TL7.0; GE Healthcare Biosciences, Uppsala, Sweden). The intensity of the bands was analyzed using ImageJ 1.52a software.

Microarray and gene expression analysis

The RNA expression data of human cirrhotic and healthy livers were collected from the GEO database (<http://www.ncbi.nlm.nih.gov/geo>). The GSE14323, GSE36411, and GSE89377 datasets contained gene expression data collected from 41, 21, and 12 cirrhotic and 19, 21, and 13 healthy liver tissues, respectively. The GEOquery R software package was used to download the GEO data and platform information. Then, the gene ID conversion was performed, and the maximum value of genes with the same name was selected. Ggplot2 package was used to plot the boxplot and density of the expression levels for each sample. Ggfortify package was used to perform the principal component analysis (PCA). DEGs between the cirrhosis and healthy liver tissue groups were identified using limma package by limiting the value of adjustment: P -value (adjust. $P < 0.05$) and the absolute value of logFC ($|\log FC| > 1.2$ or 0.6). A volcano map was generated using the ggplot2 package, and Venn diagram online tool (<http://bioinformatics.psb.ugent.be/webtools/Venn/>) was used to draw a Venn map. The DEGs were verified using the ONCOMINE server (<https://www.Oncomine.org/resource/>), which is an online available microarray database[12].

Gene ontology and pathway enrichment analysis

WebGestalt online tool (<http://www.webgestalt.org>) was used to identify DEGs enriched in biological processes, molecular functions, cellular component-related pathways, and KEGG pathways. The P -value of less than 0.05 was considered statistically significant.

PPI network construction and identification of hub genes

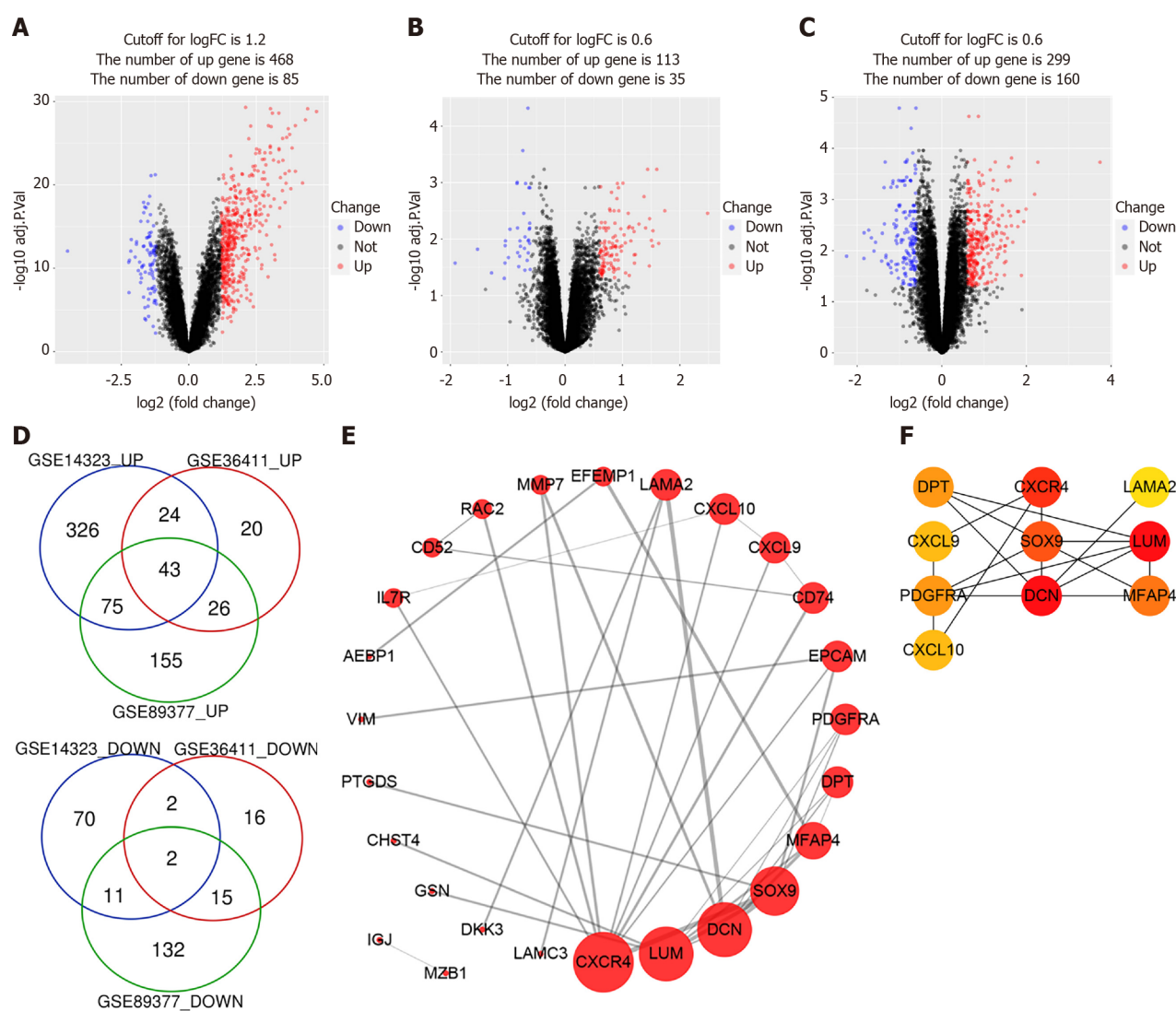
STRING online database (<https://string-db.org/>; version 11.5) was used to build the PPI network[13]. The DEGs were submitted to the STRING database to construct the PPI network. Cytoscape (version 3.7.2) was used to draw the PPI network of DEGs, and the cytoHubba plugin was used to identify hub genes[14].

Gene expression level in HCC and survival analysis

Gene expression profiling interactive analysis (GEPIA) online server (<http://gepia.cancer-pku.cn/>) was used to analyze the RNA sequencing expression data of tumors and healthy samples from the cancer genome atlas and genotype tissue expression projects[15]. We used this server to check whether the identified hub genes were differentially expressed in HCC tissues. Overall 5-year survival rates according to gene expression were obtained using the Kaplan-Meier Plotter (http://kmplot.com/analysis/index.php?p=service&cancer=liver_rnaseq)[16].

Statistical analyses

GraphPad Prism 6.0 (GraphPad Software Inc. La Jolla, CA, United States) was used for statistical analysis. Data were presented as mean $\pm$ SE or SD (for normally distributed data) or median with interquartile range (for non-normally distributed data). Statistically significant differences were determined using a two-tailed Student's t -test or analysis of variance (ANOVA). Statistical significance was set and marked as ^a $P < 0.05$, ^b $P < 0.01$, ^c $P < 0.001$, and ^d $P < 0.0001$. Replicates are indicated in the figure legends, and (n) represents the number of experimental replicates.



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Figure 1 Identification of differentially expressed genes. A-C: The volcano plots of GSE14323, GSE36411 and GSE89377. The red dots and blue dots represent up-regulated and downregulated genes, respectively; D: The Venn diagram software identified 45 common differentially expressed genes (DEGs) in three datasets (GSE14323, GSE36411 and GSE89377), including 43 upregulated genes and 2 downregulated genes; E: protein-protein interaction network of DEGs was constructed by STRING online database and drew by Cytoscape software; F: Top 10 hub genes of DEGs were identified by cytoHubba plug-in of Cytoscape and their importance were represented by their color's shade.

RESULTS

Identification of DEGs

We analyzed three gene expression datasets, GSE14323, GSE36411, and GSE89377, which included data from 74 cirrhotic and 53 healthy liver tissue samples in total. The analysis of processed sample data showed that the gene expression levels in different samples were primarily the same (Supplementary Figure 1). PCA showed that cirrhotic and healthy groups constituted individual clusters (Supplementary Figure 2). Using limma package, we extracted 553, 148, and 459 DEGs from GSE14323, GSE36411, and GSE89377 datasets, respectively (Figure 1A-C). Using Venn diagram online tool, we identified 45 DEGs (43 upregulated genes and 2 downregulated genes) associated with cirrhosis from the three datasets (Figure 1D, Table 1).

Gene Ontology and KEGG pathway analysis of DEGs

To understand the function of identified DEGs, we carried out Gene Ontology (GO) and KEGG pathways analyses using Webgestalt online server. The GO analysis results were listed according to *P*-values (Table 2), which showed that DEGs were significantly enriched in extracellular matrix organization (GO: 0030198), extracellular matrix structural constituent (GO: 0005201), collagen-containing extracellular matrix (GO: 0062023), extracellular matrix (GO: 0031012), cell adhesion (GO: 0007155), biological adhesion (GO: 0022610), and taxis (GO: 0042330). KEGG pathway analysis showed

Table 1 Gene expression profiles of GSE14323, GSE36411 and GSE89377 have 45 differentially expressed genes, including 2 downregulated genes and 43 upregulated genes in the fibrotic liver compared to normal liver

DEGs	Genes		
Upregulated	C-X-C chemokine receptor type 4 (<i>CXCR4</i>)	SH3 domain-containing YSC84-like protein 1 (<i>SH3YL1</i>)	Laminins containing the $\alpha 2$ (<i>LAMA2</i>)
	Lumican (<i>LUM</i>)	DNA-binding protein inhibitor ID-3 (<i>ID3</i>)	Microfibril-associated glycoprotein 4 (<i>MFAP4</i>)
	Prostaglandin-H2 D-isomerase (<i>PTGDS</i>)	Aldo-keto reductase family 1 member B10 (<i>AKR1B10</i>)	Marginal zone B- and B1-Cell-specific protein (<i>MZB1</i>)
	Dickkopf-related protein 3 (<i>DKK3</i>)	Ras-related protein Rac2 (<i>RAC2</i>)	Suppressor of lin-12-like protein 3 (<i>SEL1L3</i>)
	Dermatopontin (<i>DPT</i>)	Annexin A13 (<i>ANXA13</i>)	Defensin Beta 1 (<i>DEFB1</i>)
	H-2 class II histocompatibility antigen gamma chain (<i>CD74</i>)	CAMPATH-1 antigen (<i>CD52</i>)	Protein unc-93 homolog A (<i>UNC93A</i>)
	FXRD domain-containing ion transport regulator 2 (<i>FXRD2</i>)	Adipocyte enhancer-binding protein 1 (<i>AEBP1</i>)	Interleukin-7 receptor subunit alpha (<i>IL7R</i>)
	C-X-C motif chemokine 9 (<i>CXCL9</i>)	C-X-C motif chemokine 10 (<i>CXCL10</i>)	Ribonuclease pancreatic (<i>RNASE1</i>)
	SRY-Box transcription factor 9 (<i>SOX9</i>)	Gelsolin (<i>GSN</i>)	Carbohydrate sulfotransferase 4 (<i>CHST4</i>)
	Vimentin (<i>VIM</i>)	Galectin-3-binding protein (<i>LGALS3BP</i>)	Platelet-derived growth factor receptor alpha (<i>PDGFRA</i>)
	Lectin, galactoside-binding soluble 4 (<i>LGALS4</i>)	Laminin subunit gamma-3 (<i>LAMC3</i>)	Claudin-10 (<i>CLDN10</i>)
	Joining chain of multimeric IgA and IgM (<i>JCHAIN</i>)	Ectonucleotide pyrophosphatase/phosphodiesterase family member 2 (<i>ENPP2</i>)	Cholesterol 25-hydroxylase (<i>CH25H</i>)
	Apolipoprotein L3 (<i>APOL3</i>)	Decorin (<i>DCN</i>)	Complement component C7 (<i>C7</i>)
	Epithelial cell adhesion molecule (<i>EPCAM</i>)	Keratin type I cytoskeletal 23 (<i>KRT23</i>)	EGF-containing fibulin-like extracellular matrix protein 1 (<i>EFEMP1</i>)
Downregulated	Matrix metalloproteinase 7 (<i>MMP7</i>)		
	Small conductance calcium-activated potassium channel protein 2 (<i>KCNN2</i>)	Cytochrome P450 2C19 (<i>CYP2C19</i>)	

DEGs: Differentially expressed genes.

that the DEGs involved in the chemokine signaling pathway, focal adhesion, regulation of actin cytoskeleton, leukocyte transendothelial migration, pathways in cancer, and cytokine-cytokine receptor interaction, and arachidonic acid metabolism were highly enriched in patients with liver cirrhosis (Table 3).

PPI network construction and identification of hub genes

PPI network analysis aids in studying the molecular mechanisms of the disease pathogenesis. Using String v11 and Cytoscape software, we constructed a PPI network with 26 nodes and 36 edges (Figure 1E). These genes were upregulated in liver cirrhosis. The top 10 hub genes were identified using the CytoHubba plugin of Cytoscape, which included C-X-C motif chemokine ligand 9 (*CXCL9*), *CXCL10*, C-X-C motif chemokine receptor 4 (*CXCR4*), *DCN*, *DPT*, laminin subunit alpha 2 (*LAMA2*), lumican (*LUM*), microfibril associated protein 4 (*MFAP4*), platelet-derived growth factor receptor alpha (*PDGFRA*), and *SOX9* (Figure 1F).

Expression of hub genes in the liver tissue of CCl₄-induced cirrhosis mice

To study the role of the 10 hub genes in liver cirrhosis, we generated a CCl₄-induced liver cirrhosis mouse model (Figure 2A–B). The relative mRNA levels of these genes were shown in Figure 2C. Compared to those in the control group, seven genes (*CXCR4*, *DCN*, *DPT*, *LAMA2*, *LUM*, *MFAP4*, and *SOX9*) were significantly upregulated in the liver cirrhosis mice, while the expression levels of *PDGFRA*, *CXCL9*, and *CXCL10* were not significantly different between the two groups. For further validation, we performed Western blotting and immunohistochemical analysis (Figure 2D, Figure 3). These results confirmed that the protein levels of *DCN*, *DPT*, and *SOX9* were significantly upregulated in the liver tissue of cirrhotic mice.

Table 2 Gene Ontology analysis of differentially expressed genes (top 10 according to *P*-value)

Gene set	Description	Count	<i>P</i> value
GO: 0030198	Extracellular matrix organization	11	3.38E-10
GO: 0043062	Extracellular structure organization	11	1.56E-9
GO: 0005201	Extracellular matrix structural constituent	7	7.48E-8
GO: 0062023	Collagen-containing extracellular matrix	9	1.53E-7
GO: 0031012	Extracellular matrix	10	1.78E-7
GO: 0007155	Cell adhesion	15	2.78E-7
GO: 0022610	Biological adhesion	15	3.00E-7
GO: 0006935	Chemotaxis	10	0.000001284
GO: 0042330	Taxis	10	0.000001323
GO: 0005198	Structural molecule activity	11	0.000001624

Table 3 Kyoto Encyclopedia of Genes and Genomes pathway analysis of differentially expressed genes in fibrotic liver

Gene set	Description	<i>P</i> value	Genes
hsa04062	Chemokine signaling pathway	0.00	CXCR4, CXCL9, CXCL10, RAC2
hsa04510	Focal adhesion	0.01	LAMA2, LAMC3, PDGFRA, RAC2
hsa04810	Regulation of actin cytoskeleton	0.01	CXCR4, GSN, PDGFRA, RAC2
hsa04670	Leukocyte transendothelial migration	0.01	CLDN10, CXCR4, RAC2
hsa05200	Pathways in cancer	0.01	CXCR4, IL7R, LAMA2, LAMC3, PDGFRA, RAC2
hsa05416	Viral myocarditis	0.02	LAMA2
hsa04060	Cytokine-cytokine receptor interaction	0.02	CXCL9, CXCL10, CXCR4, IL7R
hsa00590	Arachidonic acid metabolism	0.02	CYP2C19, PTGDS
hsa04976	Bile secretion	0.03	FXYD2, KCNN2
hsa04024	cAMP signaling pathway	0.02	FXYD2, RAC2, SOX9

DCN, DPT and SOX9 expression and liver fibrosis progression in patients

To further explore the relationship between DCN, DPT, and SOX9 protein expression and progression of liver cirrhosis, we collected liver biopsy tissue samples from 5 healthy controls, 28 patients with CHB, and 12 patients with CHB-associated HCC. All liver tissues were divided into 5 groups (normal, S0, S1-2, S3-4, and HCC) according to METAVIR system. The clinical profile of the patients enrolled in the study is summarized in [Table 4](#). The results showed that males were the majority in S3-4 and HCC groups. The age, TBil, ALB, and CHE values in all groups and the AFP value in non-HCC group were in normal distribution, and the median AFP value in the HCC group was higher than the upper limit of normal value. In patients with CHB, HBV DNA was detected as positive, and most ALT and AST levels were elevated, which was consistent with the inflammatory activity of the liver. Most patients with HCC were detected negative for HBV DNA, which is related to antiviral treatment. Liver samples from patients with fibrosis showed increased collagen deposition, inflammatory cell infiltration, or atypical cells viewed with HE and Masson's trichrome staining ([Figure 4](#)).

Immunohistochemical results showed that the expression levels of DCN and SOX9 increased with the aggravation of liver fibrosis ([Figure 4](#), [Figure 5A](#), [Figure 5C](#)) and were significantly higher in the HCC group than those in healthy controls and fibrotic groups ([Figure 4](#), [Figure 5D](#), and [Figure 5F](#)). Further, compared with healthy controls, DPT expression was significantly increased in patients with liver fibrosis, particularly in the S3-S4 group but extremely reduced in patients with HCC ([Figure 5B](#), [Figure 5E](#)). In addition, we found that DCN was mostly expressed in the portal vein region, which was highly consistent with the distribution of collagen fibers, while SOX9 and DPT were mostly expressed in hepatocytes and several other types of cells ([Figure 4](#)).

DCN, DPT, and SOX9 expression and survival rate of patients with HCC

Due to the differential expression pattern and distribution, we decided to analyze the correlation between DCN, DPT, and SOX9 expression levels and the survival rate of patients with HCC. The GEPIA

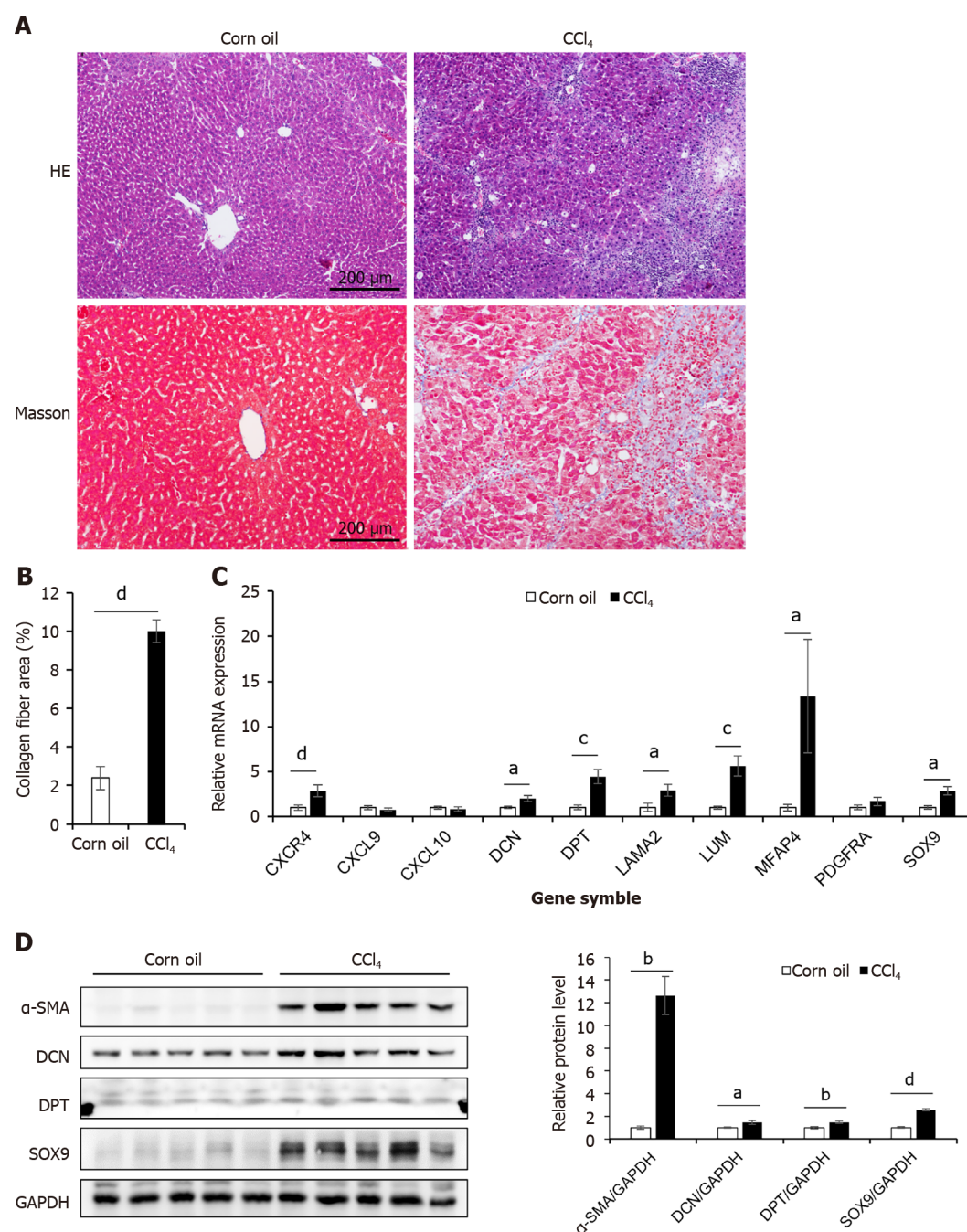


Figure 2 Expression of hub genes in the liver tissue of CCl₄-induced mouse mice. A: Masson's trichrome and HE staining of control and CCl₄-induced liver cirrhosis mouse liver tissues; B: Collagen area in Masson's trichrome staining ($n = 7$ or 8); C: The mRNA expression levels of 10 hub genes of control and CCl₄-induced cirrhosis mouse liver tissues ($n = 6$); D: The protein expression levels of α -SMA, Decorin (DCN), Dermopontin (DPT), and SRY-box transcription factor 9 (SOX9) in liver tissues of mice in two groups. The right panel showed the result of quantitative analysis ($n = 4$). All data were presented as mean $\pm$ SE. Two-tailed Student's t test were performed. ^a $P < 0.05$, ^b $P < 0.01$, ^c $P < 0.001$, ^d $P < 0.0001$.

server was used to detect the mRNA expression levels in tissues of patients 369 liver hepatocellular carcinoma (LIHC) and 160 normal tissues (Figure 6A-C). The results showed that SOX9 was significantly upregulated whereas DCN and DPT were significantly downregulated in patients with LIHC.

Kaplan-Meier curves, depicting samples from these 369 patients with HCC, showed that the 5-year survival rate of patients with high expression of SOX 9 was significantly reduced, while that of patients with high expression of DCN or DPT was increased (Figure 6D-F).

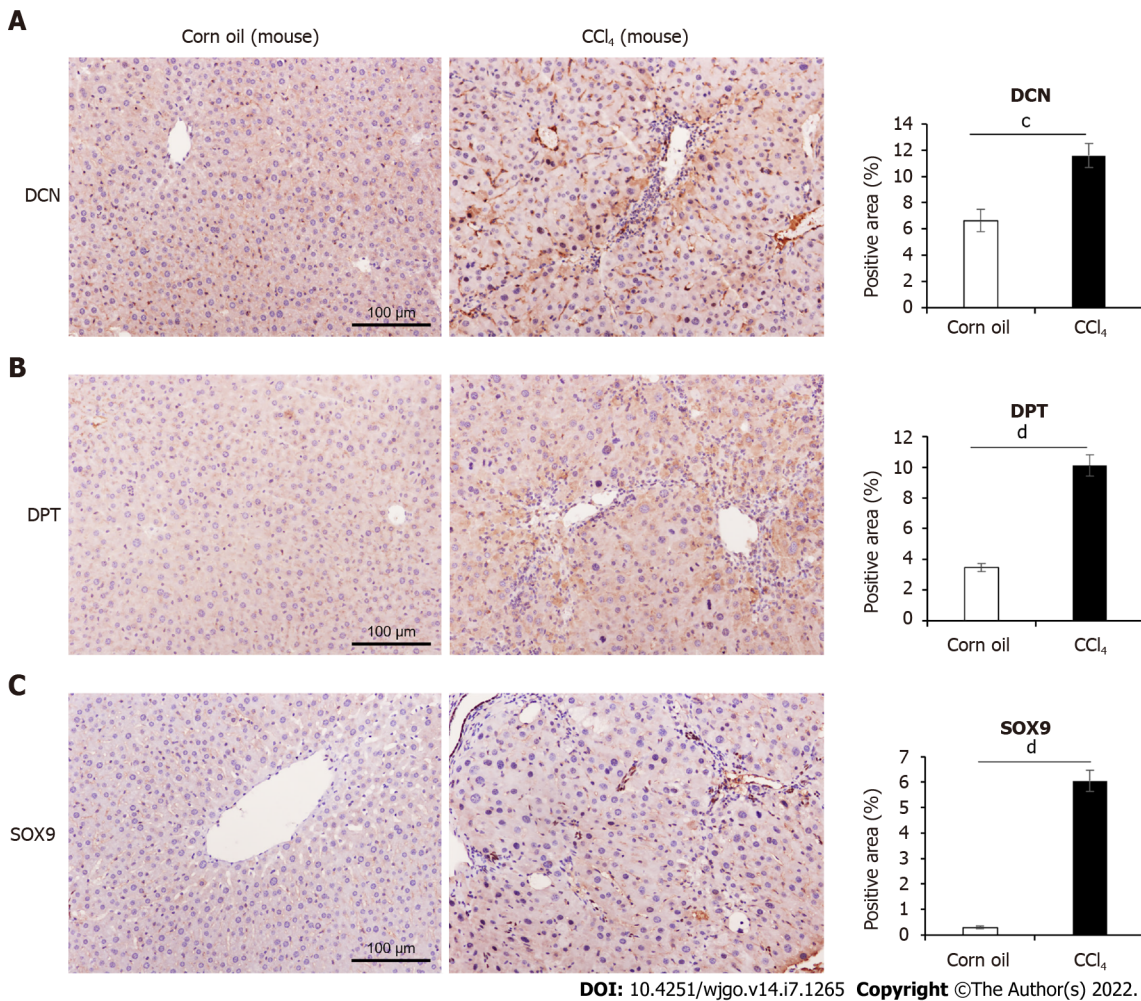


Figure 3 Comparison of decorin, dermatopontin, and SRY-box transcription factor 9 expression in CCl₄-induced mouse model. A: Immunohistochemical (IHC) analyses of the expression of Decorin (DCN) and the percentage of positive area were shown, $n = 9$ or 11 ; B: IHC analyses of the expression of Dermatoptontin (DPT) and the percentage of positive area were shown ($n = 9$ or 10); C: IHC analyses of the expression of SRY-box transcription factor 9 (SOX9) and the percentage of positive area were shown ($n = 9$ or 10). All data were presented as mean $\pm$ SE. Two-tailed Student's t test were performed. ^c $P < 0.001$, ^d $P < 0.0001$.

DISCUSSION

Fibrosis is a common pathological symptom of severe liver damage caused by various chronic liver diseases. The most common primary liver cancer, HCC, occurs most often in people inflicted with chronic liver diseases[17]. Understanding the molecular mechanisms underlying cirrhosis can help in the development of effective treatments. Although bioinformatics tools can be used to study the relationship between gene function, liver fibrosis, and HCC, it is necessary to comprehensively analyze gene expression levels and distribution in the context of disease pathology *via in vivo* experiments.

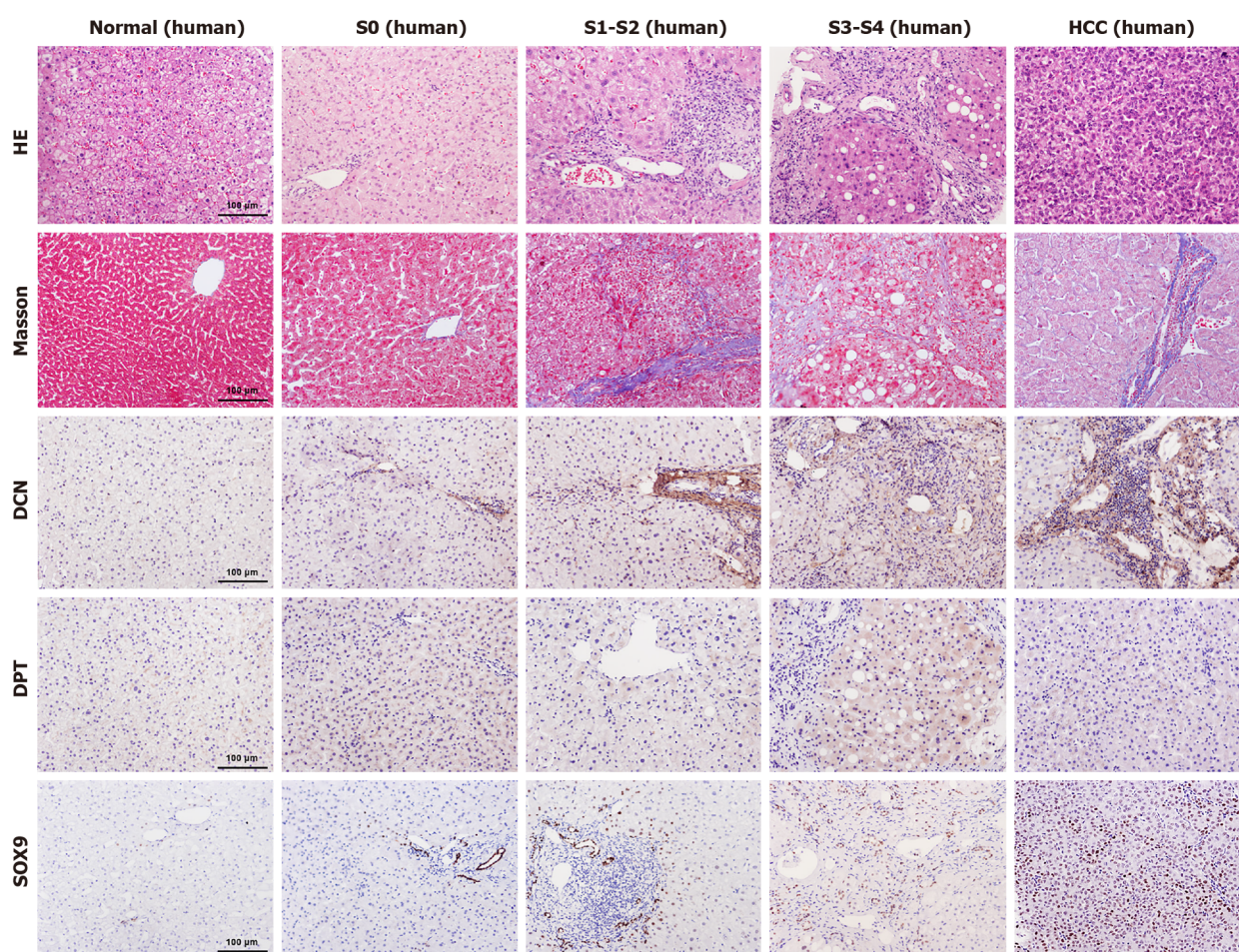
GEO is an international public resource bank for high-throughput microarray and next-generation sequencing of functional genome datasets submitted by research groups. In this study, we identified 45 DEGs from GSE14323, GSE36411, and GSE89377 datasets in the GEO database for an in-depth analysis of their biological function. Most of these DEGs have been associated with liver diseases. *CXCL9*, *ENPP2*, *CH25H*, *KRT23*, *IL7R*, *APOL3*, and *GSN* are involved in HCV or HBV infection[18-23]. *AEBP1*, *C7*, and *LUM* are overexpressed in non-alcoholic steatohepatitis (NASH)[24-26]. *CHST4*, *DEFB1*, *EFEMP1*, *MMP7*, and *SOX9* are associated with cholestasis[27-31]. *AKR1B10*, *CLDN10*, *DKK3*, *EPCAM*, *LGALS4*, and *ITM2A* are upregulated in patients with HCC[32-38]. The combined data-mining of three datasets from different sources yielded 45 DEGs, further indicating that the GEO database is indeed a useful resource for understanding the mechanism of liver diseases and using the GEO database can increase the efficiency of published resources.

Next, for detailed characterization of hub gene functions, we selected three representative genes (*DCN*, *DPT*, and *SOX9*), since their expression was consistently found to be associated with liver cirrhosis. We established a CCl₄-induced mice model and verified their mRNA and protein levels using qPCR, Western blotting, and immunohistochemistry to confirm the expression and main pathophysiological functions. The results confirmed that *DCN*, *DPT*, and *SOX9* were significantly overex-

Table 4 Clinical and histologic data for healthy controls, chronic hepatitis B and hepatocellular carcinoma patients

	Control (n = 5)	CHB-S0 (n = 4)	CHB-S1-2 (n = 13)	CHB-S3-4 (n = 11)	HCC (n = 12)
Sex (M/F), n	3/2	2/2	6/7	8/3	11/1
Age, yr, mean $\pm$ SD	41.8 $\pm$ 9.5	33.0 $\pm$ 10.3	38.2 $\pm$ 8.1	40.3 $\pm$ 6.1	52.8 $\pm$ 10.5
HBeAg(+), n	0	2	9	7	3
HBV DNA, logIU/mL, median with IQR	-	5.5 (2.4, 8.5)	5.0 (2.5, 7.0)	4.7 (2.0, 6.6)	0 (0, 3.0)
ALT, U/L, median with IQR	19.2 (16.8, 28.7)	56.6 (45.0, 75.8)	39.3 (17.9, 66.3)	51.3 (29.6, 70.0)	54.7 (24.1, 105.8)
AST, U/L, median with IQR	20.7 (19.4, 22.5)	36.3 (27.2, 44.6)	27.3 (20.9, 39.9)	31.8 (23.8, 49.7)	54.8 (27.9, 111.6)
TBil, μ mol/L, mean $\pm$ SD	18.9 $\pm$ 14.1	14.0 $\pm$ 4.3	12.8 $\pm$ 4.9	12.9 $\pm$ 4.8	16.8 $\pm$ 14.6
ALB, g/L, mean $\pm$ SD	45.9 $\pm$ 4.8	47.8 $\pm$ 4.3	46.5 $\pm$ 3.9	44.4 $\pm$ 5.0	41.3 $\pm$ 5.3
CHE, IU/L, mean $\pm$ SD	6191.3 $\pm$ 1908.0	11514.5 $\pm$ 3416.1	8887.5 $\pm$ 1964.8	7708.5 $\pm$ 2064.4	6538.7 $\pm$ 7065.7
AFP, ng/ml, mean $\pm$ SD or median with IQR	2.5 $\pm$ 1.4	2.0 $\pm$ 0.8	4.7 $\pm$ 6.1	11.3 $\pm$ 22.1	37.9 (9.3, 388.9)

F: Female; M: Male; ALT: Alanine transaminase; AST: Aspartate transaminase; TBil: Total bilirubin; ALB: Albumin; CHE: Cholinesterase; AFP: Alpha-fetoprotein; IQR: Interquartile range; HCC: Hepatocellular carcinoma; CHB: Chronic hepatitis B.



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Figure 4 Comparison of decorin, dermatopontin, and SRY-box transcription factor 9 expression in human liver tissues. The expression levels of Decorin (DCN), Dermatopontin (DPT) and SRY-box transcription factor 9 (SOX9) in normal, S0, S1-S2, S3-S4, Hepatocellular carcinoma (HCC) groups were analyzed by immunohistochemistry. The H&E staining and Masson's trichrome staining were shown also.

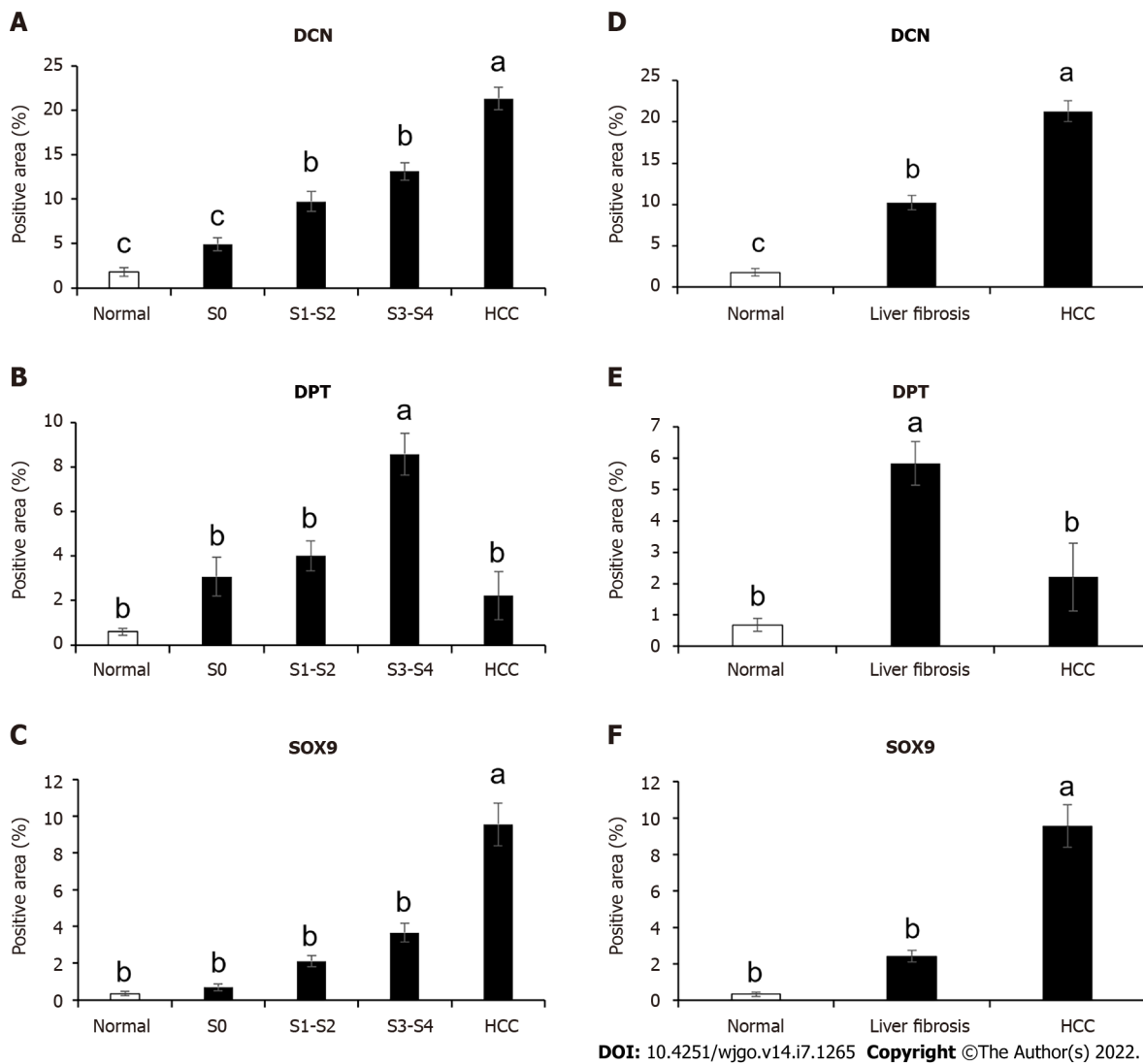


Figure 5 Expression levels analysis of decorin, dermatopontin, and SRY-box transcription factor 9 in human liver tissues. A-C: The percentage of positive area of decorin (DCN), dermatopontin (DPT) and SRY-box transcription factor 9 (SOX9) among normal ($n = 5$ or 7), S0 ($n = 3$ or 4), S1-S2 ($n = 10$ or 12), S3-S4 ($n = 8$, 9 or 11) and hepatocellular carcinoma (HCC) ($n = 11$, 12 or 14) groups were counted; D-F: The percentage of positive area of DCN, DPT and SOX9 in normal ($n = 5$ or 7), liver fibrosis ($n = 22$, 23 or 26) and HCC ($n = 11$, 12 or 14) groups were counted. All data were presented as mean $\pm$ SE. One-way ANOVA with multiple comparisons and Tukey's post-test were performed, $^aP < 0.05$, $^bP < 0.01$, $^cP < 0.001$.

pressed in human and mouse fibrotic liver; however, there were differences in the relationship between their expression levels and the survival rate of patients with HCC.

The DCN gene encodes a member of the small leucine-rich proteoglycan family of proteins, which can act as a tumor repressor in a variety of cancers[39]. DCN is a regulator of matrix assembly and not only targets transforming growth factor-beta 1 (TGF- β 1) but is also involved in the maturation of collagen fibrils[40,41]. The enhanced deposition of DCN reflects the stimulatory effect of overproduction of TGF- β 1[41]. Dudás *et al*[42] indicated that high amounts of TGF- β 1 colocalize with DCN within the fibrotic areas of the liver using a cohort of liver pathologies, including chronic hepatitis, fibrosis, and cirrhosis, which is consistent with our results. However, contrary to our results, Shang *et al*[43] found DCN mRNA expression to be downregulated and not upregulated in patients with HCC *via* gene expression profile analyses. Although GEPIA analysis showed that the mRNA expression level of DCN in ILHC was lower than that normal tissues, the heterogeneity within each group, especially the ILHC group, was very different. In addition, our results were based on immunohistochemical analysis, different from the expression profile of the whole liver tissue used by Shang *et al*[43]. From the distribution of immunohistochemical sections, we concluded that almost all the increase in DCN expression was localized in the collagen-intensive area of the portal region and not in the hepatic lobule, which indicates that the upregulated DCN significantly represents an increase in matrix assembly. These may partly explain why the increased DCN is not associated with the lower 5-year survival rate of HCC patients, and why the expression pattern of DCN expression analyzed by GEPIA server was different from our study. Considering these inconsistent results, the expression, location and function of DCN deserves further study.

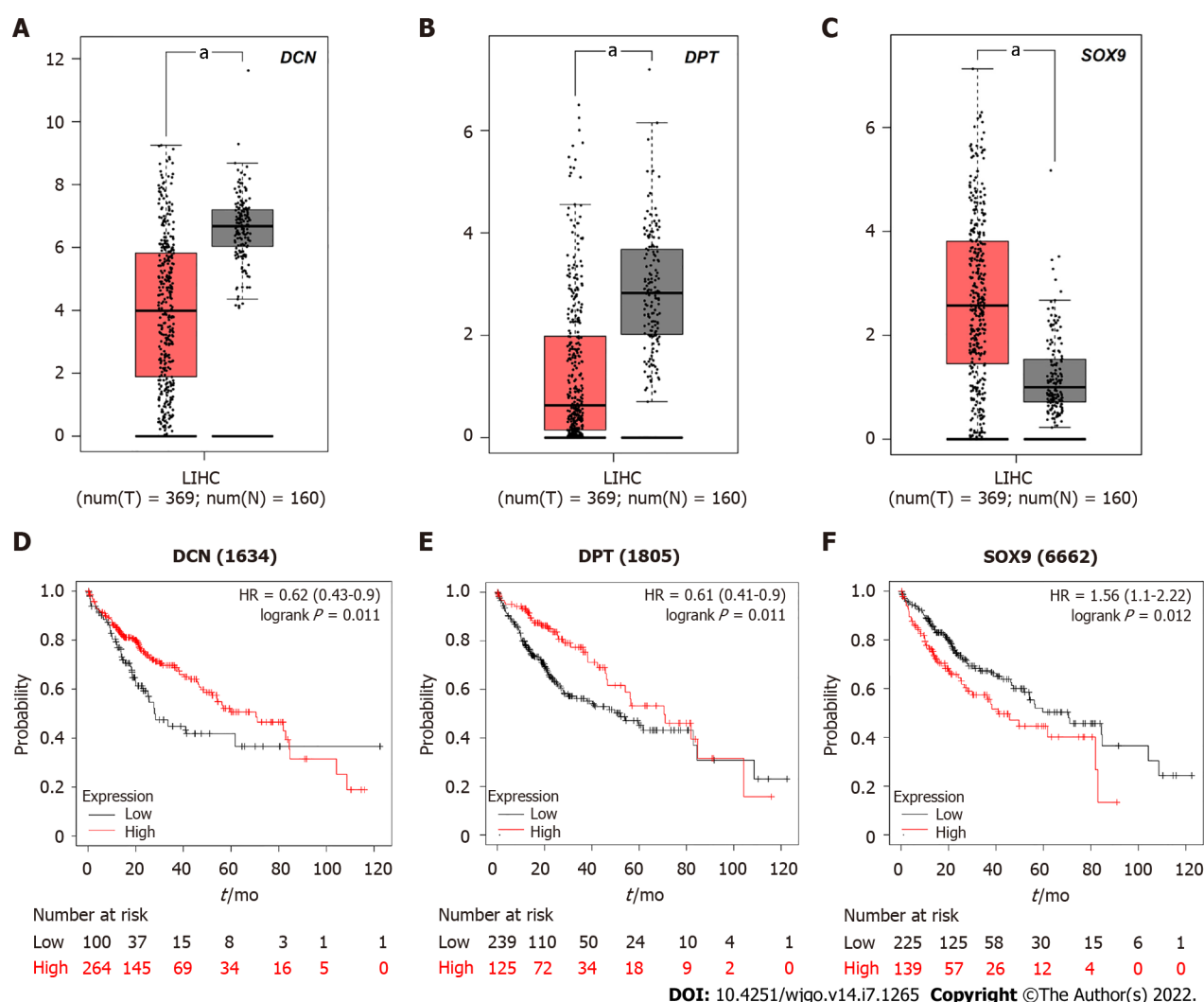


Figure 6 The relationship between decorin, dermatopontin, SRY-box transcription factor 9 expression and survival rate of hepatocellular carcinoma patients. A-C: Decorin (DCN), dermatopontin (DPT), and SRY-box transcription factor 9 (SOX9) were analyzed by gene expression profiling interactive analysis server (GEPIA) to determine their expression level differences between hepatocellular carcinoma (HCC) and normal liver tissues. Red box represents tumor tissue and gray box represents normal tissue; D-F: Prognostic information of hub genes. Kaplan-Meier plotter online tool was used to identify the prognostic information of DCN, DPT and SOX9, which associated with the survival rate of HCC patients ($^aP < 0.05$).

DPT is a downstream target of the vitamin D receptor. Fu *et al*[44] reported that mRNA expression of DPT was significantly downregulated in HCC, while its protein was weakly expressed in tumorous tissues compared to that in non-tumorous tissues. However, Lefebvre *et al*[45] suggested that DPT is upregulated in active NASH and fibrosis, and it is necessary for collagen deposition in profibrotic conditions. Our results also confirmed that the expression of DPT increased with the aggravation of liver fibrosis. Interestingly, a previous study showed that DPT interacts with DCN, which influences collagen fibrillogenesis and increases TGF- β 1 signaling[46]. Our study identified these two molecules in a combined screening, suggesting that both DPT and DCN play an important role in the occurrence and development of liver fibrosis and HCC. However, the interactions between them needs to be further studied.

During tumorigenesis, SOX9 is upregulated in various tumors and plays an essential role in tumor progression as an oncogene[29], which regulates cellular proliferation, senescence, and self-renewal and is highly expressed in liver cancer stem cells[47]. In addition, SOX9 was the earliest marker expressed by biliary precursors[48]. It has been confirmed as a transcription factor that regulates bile duct development and contributes to liver regeneration and fibrosis[47]. In this study, we confirmed that SOX9 was positively correlated with the degree of fibrosis, and the high expression of SOX9 indicated a decline in the 5-year survival rate of patients with HCC, which is consistent with the results of other studies. In addition, our results showed that SOX9 was enriched and expressed in bile duct cells in mouse and human fibrotic livers, and the expression levels of SOX9 in hepatocytes were also increased significantly in patients with HCC. This observation can be explained by the fact that SOX9 mediates the transdifferentiation of hepatocytes into bile duct epithelial cells[47]. However, the detailed molecular mechanism of SOX9 overexpression in hepatocytes requires further elucidation.

Our current study has some limitations. First, we only analyzed the transcriptome, and many studies have shown that epigenetic modifications and non-coding RNAs also play an important role in the progression of liver diseases[33,49]. Secondly, the sample size in terms of number of patients was small, and immunohistochemistry was a semi-quantitative method. In addition, although our study had identified the signal transduction pathway involved in liver cirrhosis and HCC, it lacked in-depth analysis on the mechanism of action of these molecules, which needed to be further studied using *in vivo* studies or knock-out mice.

CONCLUSION

We screened GEO databases and obtained 45 DEGs and 10 hub genes (particularly *DCN*, *DPT*, and *SOX9*) in cirrhotic liver tissues. Upregulated expression of *DCN*, *DPT*, and *SOX9* was all positively correlated with the degree of fibrosis, but there may be differences between their correlation with the 5-year survival rate of HCC patients.

ARTICLE HIGHLIGHTS

Research background

Liver fibrosis and hepatocellular carcinoma (HCC) are common adverse consequences of chronic liver injury. Establishing more effective biomarkers is important for understanding the pathogenesis, occurrence, development mechanisms of liver fibrosis and HCC, as well as to identify new diagnostic and therapeutic tools.

Research motivation

Bioinformatics has screened out many differentially expressed genes related to liver fibrosis; however, it is unknown whether these genes are different in animal and human liver fibrosis tissues, especially among the different fibrotic degrees. Therefore, we should carefully analyze the research results of bioinformatics.

Research objectives

To identify liver fibrosis-related core genes, we observed and compared the differential expression pattern of core genes in patients with liver fibrosis and HCC.

Research methods

In this study, we analyzed the expression pattern of hub genes of fibrosis and HCC. Bioinformatics analyses, quantitative polymerase chain reaction, Western blot, and immunohistochemistry of liver tissues from mouse model and patients were performed to identify novel biomarkers of liver fibrosis and HCC.

Research results

Ten hub genes (*CXCL9*, *CXCL10*, *CXCR4*, *DCN*, *DPT*, *LAMA2*, *LUM*, *MFAP4*, *PDGFRA*, and *SOX9*) associated with cirrhosis were screened from GSE14323, GSE36411, and GSE89377 datasets. *DCN*, *DPT*, and *SOX9* were highly expressed in the CCl_4 -induced mouse model of liver cirrhosis and fibrotic patient liver samples, and their expression levels were associated with the degree of fibrosis. In patients with HCC, *SOX9* was upregulated, while *DCN* and *DPT* were downregulated. However, the 5-year survival rate of HCC patients with high *SOX9* expression was significantly reduced, which is different from *DPT* or *DCN*.

Research conclusions

We screened and identified 10 hub genes related to fibrosis. The expression levels of *DCN*, *DPT*, and *SOX9* were positively correlated with the degree of liver fibrosis but showed different correlations with the survival rate of patients with HCC.

Research perspectives

The integrated approach of bioinformatics and molecular biology is more efficient to research multifactorial diseases, such as liver fibrosis and liver cancer. Future studies on the differences on *DCN*, *DPT*, and *SOX9* expression may help in the better understanding of the mechanisms involved in the development of liver fibrosis and HCC.

FOOTNOTES

Author contributions: Li Y and Yuan SL contributed equally to this work; Wang Q and Xie W designed the research and were co-corresponding authors; Yuan SL, Li Y, Yin JY, Yang K and Zhou XG performed the experiments; Yuan SL and Li Y analyzed the data; Yuan SL, Li Y and Wang Q wrote the manuscript; Yuan SL provided vital reagents and analytical tools; all authors read and approved the final manuscript.

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Informed consent statement: Patients were not required to give informed consent to the study because the analysis used anonymous clinical data and liver tissue samples.

Conflict-of-interest statement: All the authors report no relevant conflicts of interest for this article.

Data sharing statement: Publicly available datasets were analyzed in this study, which can be found here: GSE14323, GSE36411 and GSE89377. Technical appendix, statistical code, and data set available from the corresponding author at wangqidl04@ccmu.edu.cn. No additional data are available.

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