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Original Article

Analysis of key genes and related transcription factors in liver fibrosis based on bioinformatic technology

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Abstract: Objective: To analyze differentially expressed genes (DEGs) related to liver fibrosis, and clarify the key genes and the possible targets in the progression of liver fibrosis. Methods: Using microarray datasets, GSE38199 was extracted from Gene Expression Omnibus (GEO), and a bioinformatics method was performed to find DEGs and transcription factors related to liver fibrosis. Results: A total of 58 DEGs were screened out according to GEO2R online analysis tool, which included 49 up-regulated and 9 down-regulated genes. These DEGs were mainly involved in formation with the extracellular region and extracellular exosome through gene ontology (GO) enrichment analysis. Kyoto Encyclopedia of Gene and Genome (KEGG) pathway enrichment analysis showed that DEGs mainly participated in the PI3K-Akt signaling pathway, focal adhesion, ECM-receptor interaction, and metabolic pathways. Based on the results of the Protein-Protein Interaction (PPI) network and Molecular Complex Detection (MCODE) analysis, 9 key genes (*COL1A1, FBN1, BGN, COL6A3, MMP2, FBLN5, LUM, PDGFRB, LOXL1)* were screened out. A total of 30 transcription factors were found according to these 9 key genes, of which 4 transcription factors (Stat3, Trp53, NF-κB1, Sp1) were enriched. Conclusion: Stat3, Trp53, NF-κB1, and Sp1 were all related to the development of liver fibrosis, and *FBLN5* might be a target for liver fibrosis.

Keywords: Liver fibrosis, differentially expressed genes, bioinformatics

Introduction

Liver fibrosis is an intermediate process of liver cell damage and inflammatory response with various causes (such as hepatitis virus infection, alcohol abuse, immune response, drug and chemical poison damage), which may lead to chronic progressive liver disease. It has been found that during the liver injury-inflammation-repair progress, quiescent hepatic satellite cells (HSCs), normally located at the space of Disse, are activated and then trans-differentiated into myofibroblasts (MFCs). These MFCs can produce a large amount of extracellular matrix (ECM) dominated by collagen and thus lead to liver fibrosis [1-3]. The imbalance of ECM deposition and degradation in the liver is a necessary stage for the development of chronic liver injury to cirrhosis, and the main reason for progression to liver cancer [4]. It has been confirmed that the activation and proliferation of HSCs is a key step in the progression of liver fibrosis, so taking effective measures to interfere with the activation process of HSCs is of great importance for treatment of fibrosis. There are several classic animal models of liver fibrosis, such as bile duct ligation and repeated injections of carbon tetrachloride (CCL4) [5]. But these models only lead to hepatocellular carcinoma when giving additional processing factors (such as a lack of choline or carcinogens). Therefore, the author established platelet-derived growth factor C (PDGF-C) transgenic mice [6].

PDGF-C is a recently discovered member of platelet derived growth factor (PDGF) ligand family. The difference between PDGF-C and PDGF-A or PDGF-B is that PDGF-A and PDGF-B are secreted by cells as bioactive dimers, while

PDGF-C is a precursor polypeptide secreted in cells. By extracellular protein cleavage, PDGF-C produces active growth factor PDGF-CC [7-9]. Overexpression of PDGF-C in hepatocytes will induce liver fibrosis, hepatocyte steatosis, hepatoma, and even hepatocellular carcinoma. Therefore, PDGE-C transgenic mice could be used as a unique model to study progression from liver fibrosis to tumor.

At present, research on liver fibrosis mainly focuses on inhibition of the activation of HSCs and trans-differentiation of HSCs into MFCs and fibroblasts. TGF-β and PDGF are two major cytokines that promote activation of HSCs and ECM proliferation. Besides these, there are many other cytokines and intracellular signal transduction pathways involved in this process [10, 11]. Therefore, the mechanism of occurrence and development of liver fibrosis is very complicated. If we could find more specific biomarkers concerned with liver fibrosis or related mechanisms at the molecular level, it would benefit research. The purpose of this study is to use gene chip and bioinformatic technology to find molecular markers related to liver fibrosis.

Gene chips, an important technology platform in the field of life sciences, is an effective method for screening differentially expressed genes (DEGs). However, bioinformatic technology is also required to analyze and process thousands of genes on the gene chip to obtain useful information.

Materials and methods

Microarray data information

Gene Expression Omnibus (GEO), the largest public database of gene expression profiles at present, is managed and maintained by the National Center for Biotechnology Information (NCBI, https://www.ncbi.nlm.nih.gov/). The chip data GSE38199, downloaded from GEO, contains 7 cases of PDGF-C transgenic and 9 cases of wild-type mice.

Processing and analyzing of DEG data

GEO2R (https://www.ncbi.nlm.nih.gov/geo/geo2r/), an online tool, was used to compare different data sets and analyze the GSE38199 microarray data set. DEGs between PDGF-C transgenic and wild-type mice were screened out by $P < 0.05$ and $|\log FC| > 2$, then heat map analysis was performed with these DEGs on HemI (http://hemi.biocuckoo.org/down.php).

Analyzing DEGs by GO function and KEGG pathway

GO and KEGG pathway analysis were used to describe and classify a class of genes. GO refers to the basic ontology database, which can classify, define and annotate genes from three aspects: cell composition, bioinformatics process, and molecular function [12]. Moreover, KEGG can also classify genes with the same biologic function, which helps researchers to study gene expression and explore information expressed as a whole [13]. Database for Annotation, Visualization and Integrated Discovery (DAVID, https://david.ncifcrf.gov/) database was used to identify DEGs by analyzing GO function and KEGG pathway, in which $P < 0.05$ was considered significant.

PPI network construction and module selection

PPI network could effectively screen out the key proteins correlated with liver fibrosis, and analyze their relationship. The internet online tool-STRING (https://string-db.org/) database is commonly used to exploring gene correlation, which contains known and predicted protein interactions [14]. In this study, a PPI network was constructed on STRING between these DEGs. The interactions between proteins with a score ≥ 0.4 were visualized using Cytoscape. The top 10 DEGs with a high degree of connectivity in the PPI network were selected as key genes of liver fibrosis.

MCODE is a plug-in of Cytoscape, which is used to find highly connected regions in PPI networks. According to the modules selected from the PPI network, GO function and KEGG pathway enrichment analysis of the most meaningful modules were carried out using DAVID, with $P < 0.05$ considered significant.

Searching for transcription factors

Transcriptional Regulatory Relationships Unraveled by Sentence based Text mining (TRRUST, https://www.grnpedia.org/trrust/) database records the regulatory relationships of transcription factors. It contains not only the target genes corresponding to transcription factors, but also the regulatory relationships

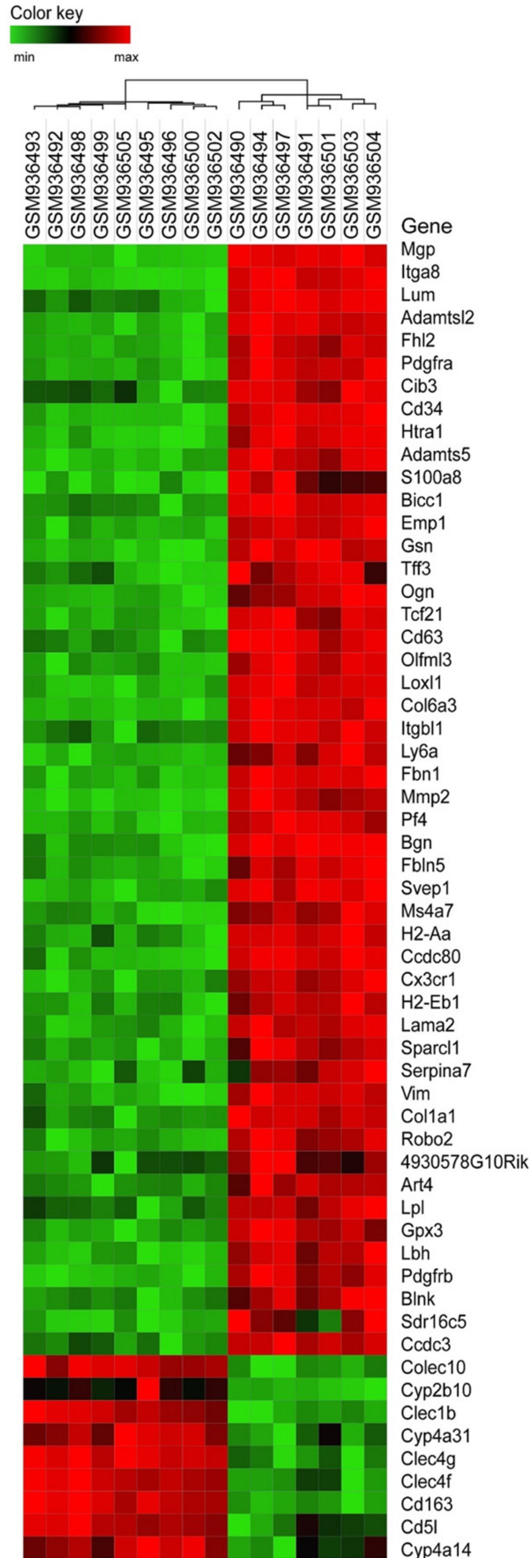

Figure 1. Heat map of DEGs. The heat map consists of 16×58 squares, each representing the level of gene expression in a sample, with high expression indicated by red and low expression indicated byf green.

between transcription factors [15]. In this study, transcription factors related to liver fibrosis were found by key genes in the TRRUST database.

Result

Identification of DEGs

In the study, 7 cases of PDGF-C transgenic mice and 9 cases of wild-type mice were used in this study. A total of 35557 genes were obtained from the GSE38199 data set, and 58 DEGs, including 49 up-regulated and 9 down-regulated genes, were screened out. Analysis of DEGs was carried out by heat map (**Figure 1**).

Results of GO functional enrichment analysis

Based on DAVID analyzing GO function of DEGs, 8 GO function enrichment results were screened out (**Table 1**). The cytological composition analysis of these DEGs showed that most of them were involved in the composition of extracellular region, extracellular exosome, and extracellular space. Biologic processes were mainly concentrated in extracellular matrix organization, Chemotaxis, and metanephric glomerular capillary formation.

Results of KEGG pathway enrichment analysis

KOBAS 3.0 software was used to analyze the KEGG pathway of DEGs. Significantly enriched pathways were screened out (**Tables 2**, **3** and **Figure 2**). KEGG pathway enrichment analysis showed that the up-regulated DEGs were mainly involved in PI3K-Akt signaling pathway, Focal adhesion, Human papillomavirus infection, ECM-receptor interaction, and Cell adhesion molecules. Down-regulated DEGs were mainly related to Metabolic pathways, Arachidonic acid metabolism, and Retinol metabolism.

Analysis of DEGs by PPI network

58 DEGs were inputted into STRING database to analyze molecular interaction. Then the data were imported into Cytoscape, and the top 10 Hub genes were found by plug-in cytoHubba (**Figure 3**).

Analysis of PPI function modules

Module analysis was conducted using MCODE, and the most significant modules of DEGs were

Table 1. GO function analysis of 8 differentially expressed genes (DEG)

Category	Term	Description	Count	*P* value
CC	GO:0005576	extracellular region	30	9.45E-17
CC	GO:0031012	extracellular matrix	15	5.15E-14
CC	GO:0005578	proteinaceous extracellular matrix	15	1.39E-13
CC	GO:0005615	extracellular space	19	6.79E-08
CC	GO:0070062	extracellular exosome	23	1.11E-06
BP	GO:0030198	extracellular matrix organization	6	1.54E-05
BP	GO:0006935	Chemotaxis	6	1.82E-05
BP	GO:0072277	metanephric glomerular capillary formation	3	2.24E-05

Table 2. KEGG pathway analysis of the top 10 up-regulated genes

Category	Term	Description	Input number	Corrected *P*-Value	*P* value
KEGG	mmu04510	Focal adhesion	6	2.78E-05	3.31E-07
KEGG	mmu04512	ECM-receptor interaction	4	0.000255332	6.69E-06
KEGG	mmu04151	PI3K-Akt signaling pathway	6	0.000255332	9.12E-06
KEGG	mmu04514	Cell adhesion molecules (CAMs)	4	0.001908865	9.09E-05
KEGG	mmu05165	Human papillomavirus infection	5	0.002184925	0.000130055
KEGG	mmu05205	Proteoglycans in cancer	4	0.002564466	0.00018317
KEGG	mmu04810	Regulation of actin cytoskeleton	4	0.002581966	0.000222585
KEGG	mmu05416	Viral myocarditis	3	0.002581966	0.000261389
KEGG	mmu05169	Epstein-Barr virus infection	4	0.002581966	0.000276639
KEGG	mmu04640	Hematopoietic cell lineage	3	0.002644313	0.000314799

Table 3. KEGG pathway analysis of the top 10 down-regulated genes

Category	Term	Description	Count	*P* value
CC	GO:0005578	proteinaceous extracellular matrix	5	3.78E-07
CC	GO:0005615	extracellular space	4	0.00393979261
CC	GO:0070062	extracellular exosome	5	0.01271927179
CC	GO:0031012	extracellular matrix	2	0.04169655023

extracted from PPI network with MCODE score = 8 (**Figure 4**). The results of the module analysis were almost the same as the 10 Hub genes obtained by PPI network analysis. Then, KEGG pathway and GO function analysis of module genes were conducted by DAVID online analysis tool, and the results are shown in **Tables 4** and **5**.

Finding related transcription factors

In the study, 9 key genes were used for searching related transcription factors in TRRUST database. TRRUST database finally identified 4 out of 9 genes, namely *COL1A1, MMP2, PDGFRB, FBLN5*, and the transcription factors contained in these 4 genes are shown in **Table 6.** Finally, 4 transcription factors were enriched, and their enrichment is shown in **Table 7.**

Discussion

In this study, bioinformatics was used to analyze the chip data GSE38199 in the GEO database. A total of 58 DEGs were screened out. In addition, PPI network and module analysis revealed 9 DEGs related to liver fibrosis: *COL1A1, FBN1, BGN, COL6A3, MMP2, FBLN5, LUM, PDGFRB and LOXL1*. 30 total transcription factors were obtained from these 9 DEGs, of which 4 transcription factors were enriched: Stat3, Trp53, NF-κB1, and Sp1.

Transcription factor specific protein 1 (Specificity protein 1, Sp1) is a nuclear transcription factor that interacts with the GC/GT box of the gene regulatory region and other proteins. It is related to initiation of transcription of many target genes. It plays a very important role in cell

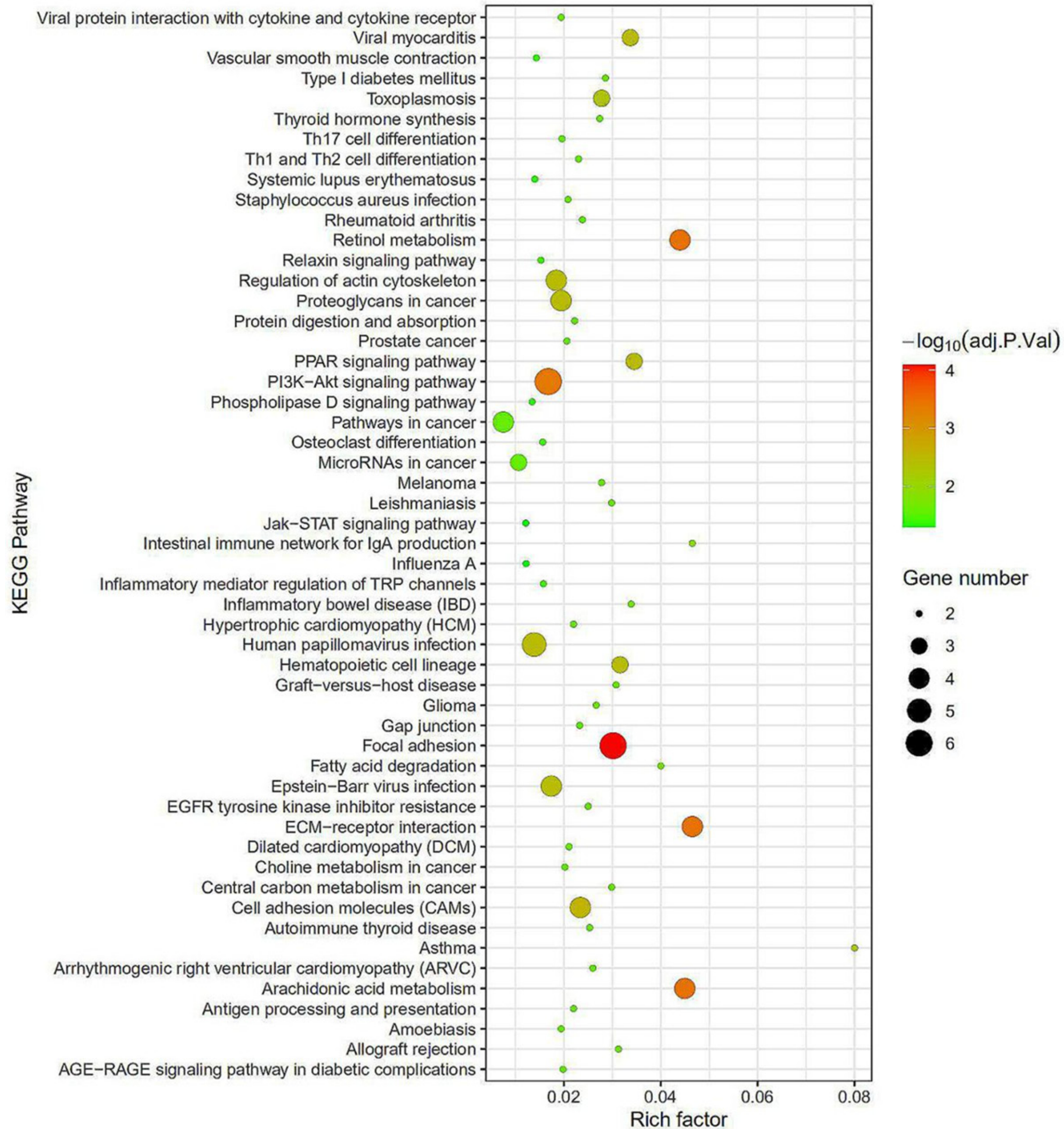

Figure 2. KEGG pathways enrichment analysis. The redder the bubble color indicates a smaller the *P* value, the larger the bubble indicates a greater the number of genes. It can be seen from the figure that the bubble on Focal adhesion is the largest, and the color is the reddest, which implies the best enrichment effect.

proliferation, apoptosis, differentiation and tumor formation [16]. Among transcription factors, Sp1 is involved in the expression of ECM genes, and regulates the expression of several genes associated with downstream targets of TGF-β. Thus it plays an important role in the progression of liver fibrosis [17]. One study showed that Sp1 Decoy ODN inhibited the activation of HSCs by down-regulating the expression of liver fibrosis-related genes, such as *PDGF-BB*, *α-SMA*, and *COL1α1* [18]. It reported that *COL1A1* promoter contains two Sp1 binding sites, and *COL1A1* expression induced by malondialdehyde in HSCs could be blocked by the mutation of the Sp1 site and the application of Sp1 inhibitors [19]. Another experiment found that there were two Sp1 binding sites on the *MMP2* promoter, and the deletion of this region would reduce 76% of the activity of *MMP2* promoter. However, extracellular signals

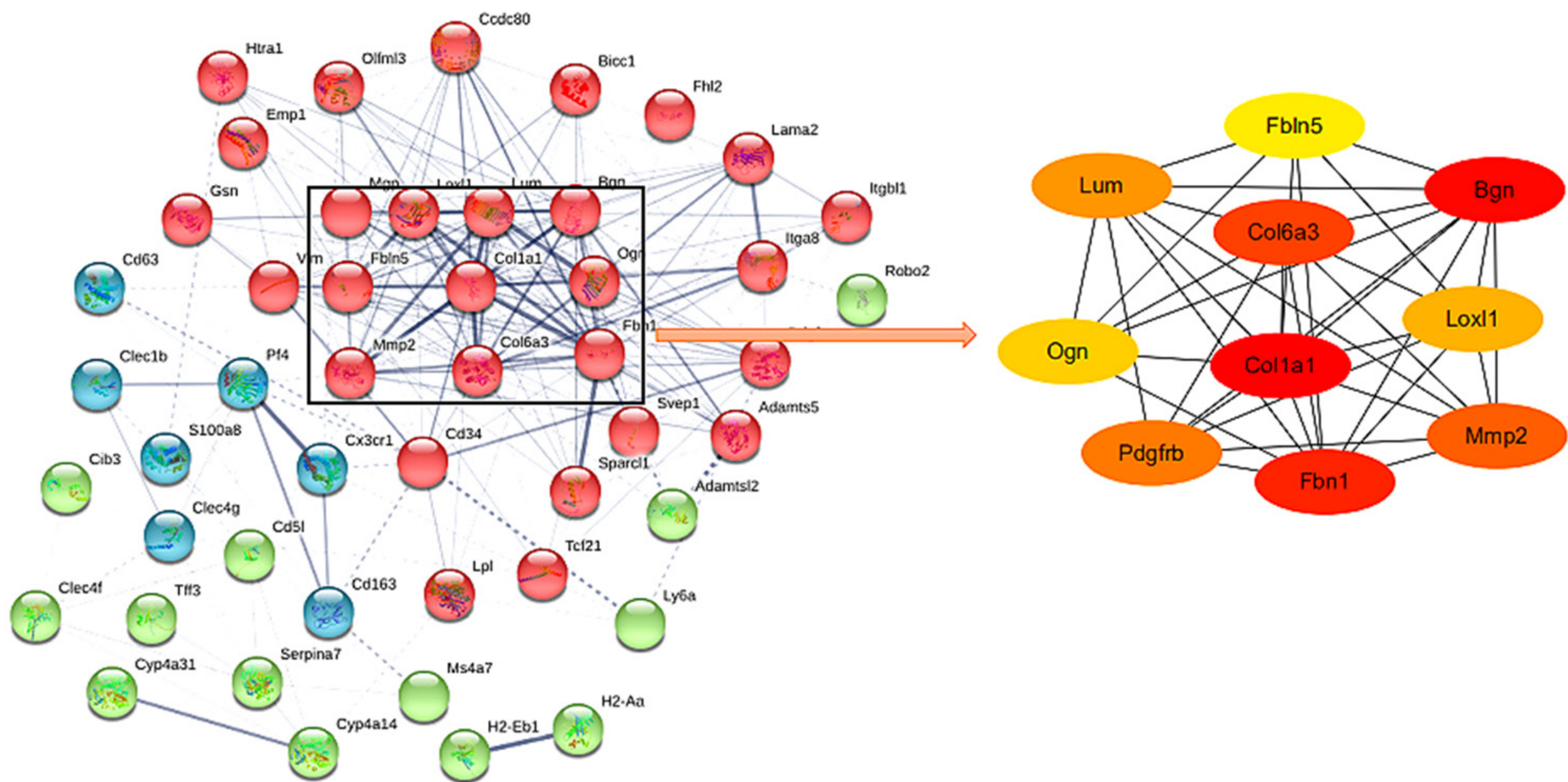

Figure 3. PPI network. Each node represents a relevant gene, and the right side is the top 10 Hub genes of the PPI network.

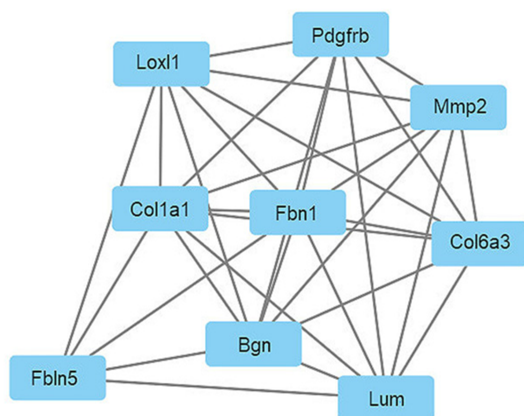

Figure 4. Significant modules in the PPI network with MCODE score = 8.

may phosphorylate Sp1 through the ERK signaling pathway to promote *MMP2* expression. These results indicate that Sp1 is closely related to liver fibrosis [20].

Transcription activating factor-3 (Stat3), an important member of Stat family, participates in cellular signal transduction and transcriptional activation, and gets continuously activated in colon cancer and gastric cancer [21]. Lee has reported that Stat3 plays a key role in the occurrence and development of fibrosis in different organs, particularly in promoting fibrosis by inducing the production of ECM. While as an inhibitor of Stat3, HJC0123 can inhibit fibrosis markers in HSCs, such as type I collagen and ECM protein fibrinogen [22]. Another Stat3 inhibitor, S3I-201, improved fibrosis in preclinical models through inhibiting the activity of Stat3 by blocking Stat3 dimerization, Stat3-DNA binding, and transcription activity [23]. Stat3 is mainly involved in the biologic processes of *COL1A1*, such as negative regulation of cell-substrate adhesion, collagen fiber organization, and collagen biosynthetic processes. Moreover, studies have proven that the expression of activated Stat3 is positively correlated with *MMP1*, *MMP2,* and *MMP10*. In addition, Stat3 had a high affinity binding site in the *MMP2* promoter region, which directly regulates the transcription of *MMP2* [24]. The Stat3 knockout mice had a higher degree of liver fibrosis than CCl4 and 3,5-diethoxycarbon-yl-1,4-dihydrocollidine (DDC)-induced wild-type liver fibrosis models [25, 26]. Moreover, activated Stat3 could stimulate liver cells to produce some unknown soluble factors, thus inhibiting the activation of HSCs and liver fibrosis. Stat3 might thus be a key signal transduction and transcription factor for regulating liver fibrosis.

NF-κB family of transcription factors, including rela (p65), NF-κB1 (p50 and p 105), NF-κB2 (p52 and p100), c-rel, and RelB, is present in almost all cell types. They can be activated by various intracellular and extracellular stimuli, such as cytokines, oxidized free radicals, and viral products [27, 28]. Activated NF-κB1 enters cells and participates in various biologic processes, such as collagen fiber organization, collagen biosynthetic process, collagen catabolic process, and positive regulation of innate immune response. Rippe and Schrum have shown that NF-κB could inhibit α1(I) collagen gene expression and localized this inhibitory activity within the promoter region (within 220 bp of the transcription start site) of the α1(I) collagen gene [29].

Trp53 (Tp53 or p53), is a transcription factor and tumor suppressor, whose protein level and post-translational modification state can be changed due to cellular stress (such as hypoxia, or DNA, and spindle damage). Activation of Trp53 causes cell apoptosis while its inactivation may lead to tumors, and mutation or inactivation of Trp53 occur in more than 50% of tumors. Trp53 not only plays an important role in tumor diseases, but also participates in the development of chronic liver disease. Weng and Yang have detected mild fibrosis in the liver of adult *Alb-Mcl-1-/-* mice, and the loss of Trp53 exacerbated the damage of liver in *Alb-Mcl-1-/- mice,* along with enhanced occurrence and severity of liver fibrosis [30]. In a CCL4-induced mouse liver fibrosis model, deletion of Trp53 in HSCs increased fibroblast proliferation and ECM deposition, which finally enhanced liver fibrosis [31]. Trp53 also participates in the positive regulation of collagen biosynthetic process, platelet-derived growth factor receptor-beta signaling pathway, and platelet-derived growth factor receptor signaling pathway. Through its target gene *PDGFRB*, Trp53 participates in the positive regulation of HSCs activation. All these findings indicate that Trp53 plays an important role in the development of liver fibrosis.

Among these target genes, the *COL1A1* encodes the largest protein connectivity, which

Table 4. Submodule GO analysis

Category	Term	Description	Input number	Corrected *P*-Value	*P* value
KEGG	mmu00590	Arachidonic acid metabolism	3	6.86E-06	1.29E-06
KEGG	mmu00830	Retinol metabolism	3	6.86E-06	1.37E-06
KEGG	mmu00071	Fatty acid degradation	2	0.000237189	7.12E-05
KEGG	mmu03320	PPAR signaling pathway	2	0.000522879	0.000209151
KEGG	mmu04750	Inflammatory mediator regulation of TRP channels	2	0.000877398	0.000438699
KEGG	mmu04270	Vascular smooth muscle contraction	2	0.000885118	0.000531070
KEGG	mmu01100	Metabolic pathways	3	0.006852979	0.004797085
KEGG	mmu00140	Steroid hormone biosynthesis	1	0.025751782	0.021968794
KEGG	mmu05204	Chemical carcinogenesis	1	0.025751782	0.023176604
KEGG	mmu04625	C-type lectin receptor signaling pathway	1	0.027513751	0.027513751

Table 5. Submodule KEGG analysis

Category	Term	Description	Input number	Corrected *P*-Value	*P* value
KEGG	mmu04510	Focal adhesion	3	0.00035532121	1.95E-05
KEGG	mmu05205	Proteoglycans in cancer	3	0.00035532121	2.15E-05
KEGG	mmu04151	PI3K-Akt signaling pathway	3	0.00092345123	0.00010922
KEGG	mmu05165	Human papillomavirus infection	3	0.00092345123	0.00011193
KEGG	mmu04512	ECM-receptor interaction	2	0.00153386492	0.00025518
KEGG	mmu04974	Protein digestion and absorption	2	0.00153386492	0.00027888
KEGG	mmu04933	AGE-RAGE signaling pathway in diabetic complications	2	0.00164721576	0.00034940
KEGG	mmu04926	Relaxin signaling pathway	2	0.00239796570	0.00058132
KEGG	mmu05200	Pathways in cancer	2	0.03294246829	0.00898430
KEGG	mmu05219	Bladder cancer	1	0.03776680097	0.01144448

Table 6. Distribution of transcription factors in 9 key genes

TF	Target	TF	Target	TF	Target
Nfic	Col1a1	Ep300	Mmp2	Trp53	Mmp2
Nfkb1	Col1a1	Fos	Mmp2	Smad2	Fbln5
Prdm5	Col1a1	Fosb	Mmp2	Smad3	Fbln5
Rela	Col1a1	Fosl1	Mmp2	Myc	Pdgfrb
Runx2	Col1a1	Jun	Mmp2	Trp53	Pdgfrb
Smad1	Col1a1	Junb	Mmp2	Trp73	Pdgfrb
Sox8	Col1a1	Jund	Mmp2		
Sp1	Col1a1	Nfkb1	Mmp2		
Stat3	Col1a1	Snai1	Mmp2		
Usf1	Col1a1	Sp1	Mmp2		
Usf2	Col1a1	Srf	Mmp2		
Yy1	Col1a1	Stat3	Mmp2		

suggests its core position in the network. Overexpression of collagen is the basic feature of liver fibrosis, and an important cause of organic pathologic change of organs. Besides, liver fibrosis is mainly characterized by abnormal accumulation of extracellular matrix components which mainly composed of type I collagen [32]. Thus, inhibiting the expression of type I collagen is one of the current strategies to treat liver fibrosis. When HSCs are activated, the expression of MMPs increases, along with the increased expression of TIMPs, the inhibitor of MMPs. If TIMPs increase too fast, the ratio of MMPs/TIMPs will change, which makes an imbalance of synthesis and degradation of ECM, and promotes the development of liver fibrosis [33, 34]. During this process, the expression of *MMP2* also increased significantly, which closely related to proliferation of activated HSCs. So inhibition of *MMP2* activity is a choice to treat liver fibrosis. In general, *PDGFRB* in liver is minimally expressed, but significantly expressed in injured liver cells, thus it might have an important role in promoting fibrosis [35]. Studies have shown that one of the targets of RTK inhibitor imatinib mesylate (Gleevec) is *PDGFRB*, which can inhibit the activation of HSCs and reduce fibrosis [36]. At the

Table 7. Distribution of 4 enriched transcription factors

Key TF	Description	overlapped genes	*P* value	Q value	List of overlapped genes
Stat3	signal transducer and activator of transcription 3	2	0.000657	0.00263	Col1a1, Mmp2
Trp53	transformation related protein 53	2	0.00289	0.00548	Pdgfrb, Mmp2
Nfkb1	nuclear factor of kappa light polypeptide gene enhancer in B cells 1, p105	2	0.00411	0.00548	Mmp2, Col1a1
Sp1	trans-acting transcription factor 1	2	0.00736	0.00736	Mmp2, Col1a1

same time, *PDGFRB* transcription factor Trp53 is also involved in the positive regulation of activated HSCs, which indicates that its antagonistic role against *PDGFRB* in anti-fibrosis has become an attractive research direction.

Liver damage and an inflammatory reaction result in excessive ECM deposition in the liver, which leads to liver fibrosis. The main components of ECM include collagen, elastase, and fibronectin [37]. However, much attention has been given to the function of collagen in liver fibrosis, and less attention to the role of elastin. As an ECM protein, FBLN5 plays an important role in composition of elastic fibers, and it has been reported that the loss of FBLN5 not only decreases tissue stiffness, but also diminishes the inflammatory response and abrogates the fibrotic phenotype in a mouse cutaneous fibrosis model [38]. ECM remodeling is related to liver fibrosis. Bracht has found that the expression of *FBLN5* at the transcription and protein level increased significantly with the progression of the fibrosis stage in patients [39]. It was also noted that the expression of *FBLN5* in a NAFLD group is significantly higher than in other groups. According to bioinformatic analysis, Yuan found that *FBLN5* was the most up-regulated gene in NAFLD. GO analysis and KEGG pathway analysis also showed that *FBLN5* was involved in ECM tissue [40]. Normally, Smad2 protein exists in the cytoplasm. When phosphorylated by the activation of TGF-β1, it will move to the nucleus, and initiate the transcription of ECM and other related genes in nucleus. After that the phosphorylated Smad2 would dephosphorylate, and return to the cytoplasm. Smad2 shuttling between the cytoplasm and nucleus would transmit a fibrogenic signal, which ultimately promotes the production of ECM proteins that accelerate liver fibrosis [41, 42]. Smad3 can inhibit the activation of HSCs through the TGF-β signaling pathway and further inhibit the occurrence and development of liver fibrosis [43]. As the target gene of Smad2 and Smad3, *FBLN5* plays an important role in the occurrence and development of liver fibrosis. Thus *FBLN5* may be considered as a possible target gene for liver fibrosis treatment. However, how to regulate and control the relationship between *FBLN5* and Smad2 and Smad3 in liver fibrosis requires further research. The process of liver fibrosis is very complicated, and usually involves changes at the gene transcription level. So research regarding transcription factors and signaling pathways related to liver fibrosis is needed in gene therapy for liver fibrosis.

Conclusions

Based on the results of PPI network analysis and module analysis related to liver fibrosis, 9 key genes were obtained, of which *COL1A1*, *MMP2*, *PDGFRB* and their transcription factors Stat3, Trp53, NF-κB1, and Sp1 were associated with the occurrence and development of liver fibrosis. Related studies have confirmed that *COL1A1*, *MMP2,* and *PDGFRB* were commonly used as targets for the treatment of liver fibrosis. Smad2 and Smad3 whose target gene is *FBLN5*, are closely related to the development of liver fibrosis. Thus *FBLN5* appears to be a target for the treatment of liver fibrosis.

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Disclosure of conflict of interest

None.

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