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Multi-omic analysis identifies the molecular mechanism of hepatocellular carcinoma with cirrhosis

Mengjuan Xuan[1,4], Xinyu Gu[2,4] & Huiwu Xing[3]✉

Hepatocellular carcinoma with cirrhosis promotes the advancement of malignancy and the development of fibrosis in normal liver tissues. Understanding the pathological mechanisms underlying the development of HCC with cirrhosis is important for developing effective therapeutic strategies. Herein, the RNA-sequencing (RNA-seq) data and corresponding clinical features of patients with HCC were extracted from The Cancer Genome Atlas (TCGA) database using the University of California Santa Cruz (UCSC) Xena platform. The enrichment degree of hallmarkers for each TCGA-LIHC cohort was quantified by ssGSEA algorithm. Weighted gene co-expression network analysis (WGCNA) revealed two gene module eigengenes (MEs) associated with cirrhosis, namely, MEbrown and MEgreen. Analysis of these modules using AUCell showed that MEbrown had higher enrichment scores in all immune cells, whereas MEgreen had higher enrichment scores in malignant cells. The CellChat package revealed that both immune and malignant cells contributed to the fibrotic activity of myofibroblasts through diverse signaling pathways. Additionally, spatial transcriptomic data showed that hepatocytes, proliferating hepatocytes, macrophages, and myofibroblasts were located in closer proximity in HCC tissues. These cells may potentially participate in the process of stimulating myofibroblast fibrotic activity, which may be related to the development of liver fibrosis. In summary, we made full use of multi-omics data to explore gene networks and cell types that may be involved in the development and progression of cirrhosis in HCC.

Keywords Hepatocellular carcinoma, Cirrhosis, Multi-omics, WGCNA, inferCNV

Abbreviations

HCC	Hepatocellular carcinoma
RNA-seq	RNA-sequencing
TCGA	The Cancer Genome Atlas
UCSC	University of California Santa Cruz
WGCNA	Weighted gene co-expression network analysis
MEs	Module eigengenes
HSCs	Hepatic stellate cells
ROS	Reactive oxygen species
TME	Tumor microenvironment
ECM	Extracellular matrix
EMT	Epithelial–mesenchymal transition
FPKM	FRAGMENTS per kilobase of transcript per million mapped reads
MsigDB	Molecular signatures database
DAVID	Database for annotation, visualization, and integrated discovery
UMAP	Uniform manifold approximation and projection
LIHC	Liver hepatocellular carcinoma
BMI	Body mass index

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scRNA-seq	Single-cell RNA sequence
FGF	Fibroblast growth factor
HGF	Hepatocyte growth factor
BMP	Bone morphogenetic protein
FGFRs	Fibroblast growth factor receptors
inferCNV	Inferred copy number variation analysis
CNVs	Copy number variations
VEGF	Vascular endothelial growth factor
PME	Pre-malignant microenvironment
α-SMA	Alpha-smooth muscle actin
TIMP 1	Tissue inhibitor of metalloproteinase 1
GEO	Gene expression omnibus

HCC is ranked as the sixth most prevalent malignant tumor and the fourth highest contributor to cancer-related mortality worldwide[1]. Individuals at high risk for HCC should undergo regular monitoring to facilitate tumor detection in the early stages. However, the predominant risk factors of HCC are partly associated with the geographical region. In developing regions of Africa and Asia, infection with hepatitis B virus and exposure to aflatoxin B1 are the primary risk factors. While in developed countries and regions, hepatitis C virus infection, alcohol abuse, and metabolic syndrome are the primary risk factors[2]. Moreover, the pathogenesis of HCC has not been comprehensively elucidated. These limitations hinder the accurate diagnosis and effective treatment of HCC, eventually leading to a poor prognosis.

Notably, a majority of patients with primary HCC have a history of chronic liver fibrosis, suggesting that cirrhosis plays a crucial role in the precancerous condition of HCC, and may be one of the potential targets in the management of HCC[3]. The occurrence and development of cirrhosis are characterized by the activation of hepatic stellate cells (HSCs), persistent inflammation, excessive reactive oxygen species (ROS) production, DNA damage, and destruction and regeneration of hepatocytes[4]. Repeated destruction–regeneration events induce replication-associated mutations in hepatocytes, leading to the onset of HCC[4,5]. In addition to hepatocytes, certain non-parenchymal cells can promote the development of a tumor microenvironment (TME) through various mechanisms, thereby facilitating tumor growth. In the context of cirrhosis, injured hepatocytes alongside infiltrating immune cells can persistently stimulate HSCs, leading to the trans-differentiation of HSCs into myofibroblasts that produce collagen type I[6,7]. Myofibroblasts secrete various mediators, including extracellular matrix (ECM) proteins, cytokines, and growth factors, which interact with neighboring cells in TME, contributing to processes such as epithelial–mesenchymal transition (EMT), suppression of immune responses, and formation of new blood vessels[8]. These processes collectively promote HCC growth and metastasis.

The activation of HSCs by HCC cells within the microenvironment further induces injury to normal hepatocytes and even promotes the development of fibrosis in normal liver tissue. Numerous studies have demonstrated that HCC cells can stimulate the activation and the differentiation of HSCs into myofibroblasts, subsequently contributing to the development of fibrosis, cirrhosis, and the progression of HCC[9–11]. HSCs transition from a quiescent state to an activated phenotype, a process involving in the upregulation of alpha-smooth muscle actin (α-SMA), enhanced cellular proliferation, and increased secretion of extracellular matrix components[12]. HCC cells increasing the expression of α-SMA, which allows HSCs to acquire an activated phenotype characterized by cytoskeletal remodeling[13]. Therefore, liver fibrosis and cirrhosis are significant factors that facilitate the onset and advancement of HCC, while HCC subsequently leads to the development of fibrosis in adjacent normal hepatic tissue in turn. A crucial aspect of liver fibrosis involves the activation and differentiation of HSCs into myofibroblasts under the regulation of immune cells and malignant cells[14,15]. The above studies suggested that based on the similarities in immune microenvironment and signaling pathway regulation, the development of liver fibrosis and HCC may complement each other. However, the specific molecular mechanisms through which immune and malignant cells contribute to the development of cirrhosis remain unclear.

In this study, we analyzed the gene expression and clinical data of patients with cirrhosis and HCC from The Cancer Genome Atlas (TCGA) database to identify gene modules associated with liver cirrhosis and explored the regulatory mechanism of liver fibrosis in HCC. Based on the single-cell RNA-seq (scRNA-seq) data and the spatial transcriptomic data of HCC, we further analyzed the cell subtypes closely related to liver fibrosis, and analyzed the mechanisms related to the liver fibrosis process according to the ligand-receptor pairs.

Materials and methods

Acquisition of RNA-seq data from TCGA-LIHC

RNA-seq data were extracted from the TCGA- liver hepatocellular carcinoma (LIHC) cohort using the UCSC Xena platform (https://xena.ucsc.edu/) and quantified as $\log_2(\text{FPKM}+1)$. In the above process, fragments per kilobase (kb) of transcript per million mapped reads (FPKM) are a standard method used to normalize transcript levels in RNA-seq[16,17]. Additionally, the clinical data of each patient with HCC, including the severity of liver fibrosis, albumin levels, prothrombin time, fetoprotein levels, body mass index (BMI) and vascular tumor type, were extracted from the TCGA-LIHC cohort.

Quantification of the enrichment degree of hallmark genes in TCGA-LIHC

50 hallmark genes and their related gene symbols were obtained from the Molecular Signatures Database (MsigDB, https://www.gsea-msigdb.org/gsea/msigdb/)[18,19]. Subsequently, the *ssGSEA* algorithm in the *GSVA* package was used to calculate the enrichment scores of hallmark genes in each sample in the TCGA-LIHC cohort.

Weighted gene co-expression network analysis (WGCNA)

Based on the similarity of expression patterns of genes involved in the same pathway or biological process, co-expressed gene networks related to different degrees of liver fibrosis in HCC were constructed using the WGCNA method. According to the scale-free network standards, β within a range of 1–20 (the appropriate soft threshold) was screened using the *pickSoftThreshold* function in the *WGCNA* package[20]. Thereafter, the *blockwiseModules* function was used for one-step network construction and module detection. Finally, the correlations between module eigengenes (MEs) and liver fibrosis were examined.

Functional annotation of gene sets

The gene sets of interest were uploaded to the DAVID database (https://david.ncifcrf.gov/tools.jsp), to enrich significant biological processes ($P < 0.05$).

Pre-processing of single-cell RNA-seq (scRNA-seq) data

The scRNA-seq dataset (GSE14961) containing 10 HCC samples was downloaded from the Gene Expression Omnibus (GEO) database (https://www.ncbi.nlm.nih.gov/gds/)[21]. The *Read10X* function in the *Seurat* package was used to identify cells with 200–8000 total genes and < 20% of mitochondrial genes. Subsequently, the *SCTransform* function was used for data normalization, the *RunPCA* function was used for principal component analysis (PCA), and the *Harmony* package was used to remove batch effects between different samples. The first 30 principal components were selected to dimensionality reduction using the uniform manifold approximation and projection (UMAP)[22]. And cell subpopulations were clustered using the *FindNeighbors* and *FindClusters* functions (resolution = 0.1)[23]. Eventually, we annotated each cell type based on marker genes obtained from the *CellMarker2.0* database (http://bio-bigdata.hrbmu.edu.cn/CellMarker)[24].

Quantification of the enrichment degree of genes at the single-cell level

We calculated the enrichment scores of gene sets of interest in each cell using the *AUCell* package, with higher scores indicating higher activity of the gene set in a cell.

Cell–cell communication analysis

We used the *CellChat* package to analyze, quantify, and visualize cell-cell communications between different types of cells and myofibroblasts[25]. The *creatCellChat* function was used to establish objects, and the *identifyOverexpressedGenes* and *identifyOverexpressedInteractions* functions were used to identify ligand–receptor pairs overexpressed in cell subpopulations. Furthermore, the expression values of the above pairs were mapped to a protein-protein interaction network using the *projectData* function, and the probability of ligand-receptor interactions between different cell subpopulations was evaluated using the *computeCommunProb* function. Additionally, the *computeCommunProbPathway* function was used to evaluate the probability of each ligand-receptor interaction in a pathway in different cells. The *netVisual_bubble* function was used to generate bubble plots for visualization.

Inferred copy number variation (inferCNV) analysis

The *inferCNV* package was used to examine large-scale copy number variations (CNVs) in scRNA-seq data, and determine the intensity of gene expression in different genomic regions[26]. In this study, we used *inferCNV* to characterize the CNVs landscape including hepatocytes, epithelial cells, and proliferative hepatocytes in the genome of HCC tissues, with B cells serving as a reference. The parameters were set as follows: cluster_by_groups = TRUE, analysis_mode = 'subcluster', HMM_type = 'i3', denoise = TRUE, HMM_report_by = 'subcluster', HMM = 'TRUE'.

Quantification of different cell types based on spatial transcriptomics data

The spatial transcriptomic dataset (GSM7021870) was downloaded from the GEO database. The *FindTransferAnchors* and *TransferData* functions of the *Seurat* package were used to evaluate the proportion of different cell types in each spot.

Statistical analysis

The *Kruskal-Wallis* test was used to compare data between more than three groups of continuous variables. $P < 0.05$ was considered statistically significant. In this study, all statistical analyses were performed using the *R* software (version 4.3.1, https://www.r-project.org/) .

Results

Clinicopathologic characteristics of HCC patients with cirrhosis

Based on the degrees of liver fibrosis, the patients in the TCGA-LIHC cohort were divided into five groups as follows: no fibrosis, portal fibrosis, fibrous septa, nodular formation and incomplete cirrhosis, and established cirrhosis (Fig. 1A). We analyzed the relationship between the degree of liver fibrosis and various clinicopathologic features to demonstrate the correlation between liver fibrosis and HCC progression. (Table. S1). In particular, the established-cirrhosis group had the second highest proportion of HCC patients. Albumin levels were lower in the established-cirrhosis group than in the other four groups, indicating the fact that HCC patients with cirrhosis had low plasma levels (Fig. 1B). In addition, the prothrombin time was significantly shorter in the fibrous septa, nodular formation and incomplete cirrhosis, and established cirrhosis groups than in the no-fibrosis group (Fig. 1C). However, no significant differences were observed in terms of fetoprotein levels, body mass index (BMI), and vascular tumor type among the five groups (Fig. 1D–F).

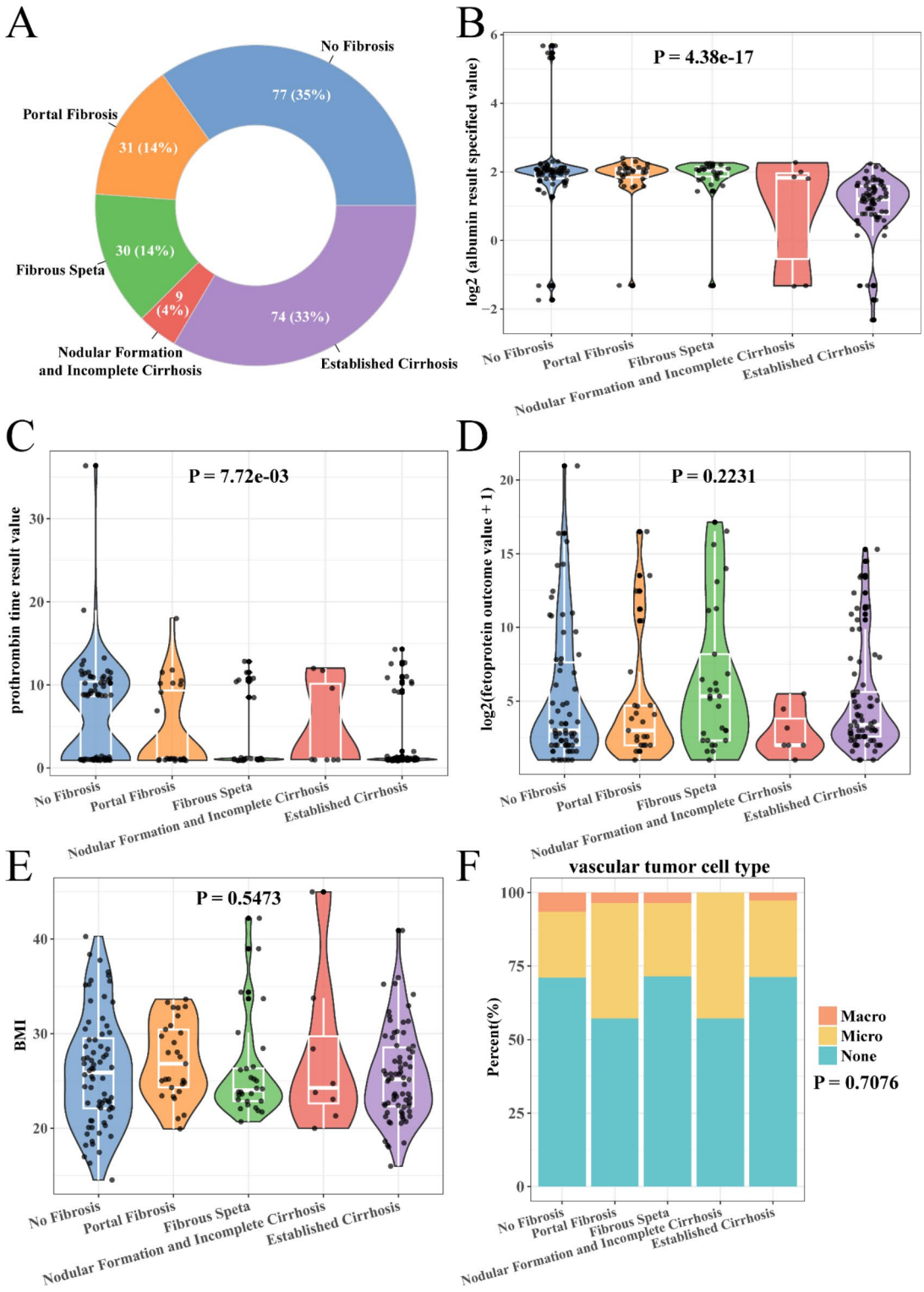

Fig. 1. Clinical characteristics of patients with HCC with cirrhosis in the TCGA-LIHC cohort. (**A**) The proportion of HCC patients with different degrees of cirrhosis. (**B**–**F**) Comparison of albumin levels (**B**), prothrombin time (**C**), fetoprotein levels (**D**), BMI (**E**), and vascular tumor type (**F**) among HCC patients with different degrees of cirrhosis.

Enrichment scores of hallmark gene sets in patients with HCC with cirrhosis

To identify cancer-related pathways enriched in different stages of cirrhosis, we calculated the enrichment scores of 50 hallmark gene sets in each patient in the TCGA-LIHC cohort using the *ssGSEA* algorithm (Fig. 2A). The results showed that patients in the nodular-formation-and-incomplete-cirrhosis group had the highest

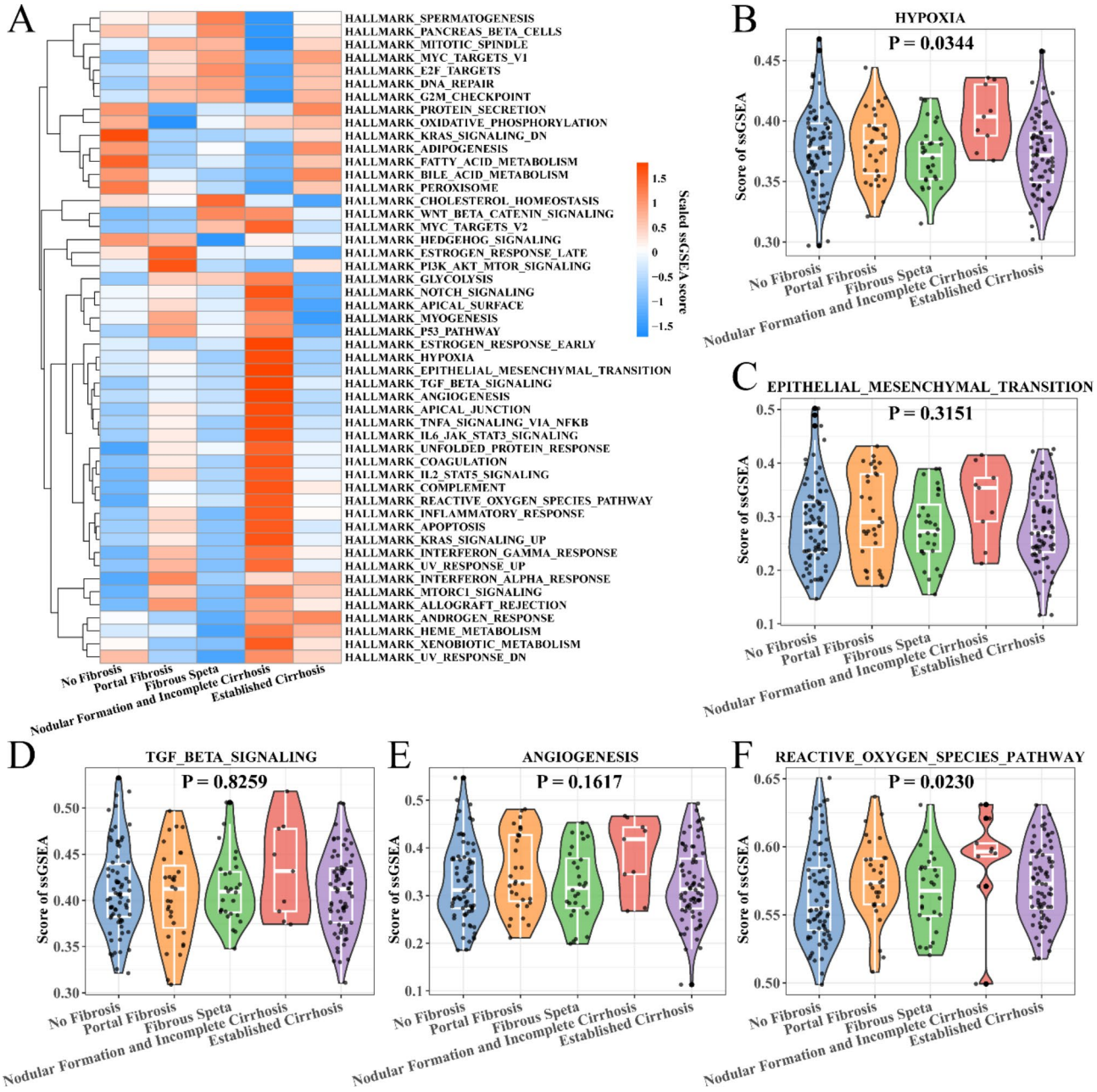

Fig. 2. Enrichment levels of hallmark gene sets in patients with HCC with cirrhosis. (**A**) Heatmap demonstrating the enrichment levels of 50 hallmark gene sets for each cirrhosis degree. (**B**–**F**) Box plots demonstrating the enrichment levels of hypoxia (**B**), epithelial-to-mesenchymal transition (**C**), TGF-β signaling pathway (**D**), angiogenesis (**E**), and ROS (**F**) enrichment for each cirrhosis degree.

enrichment scores of numerous classical hallmarks of cancer, such as hypoxia, epithelial-mesenchymal transition (EMT), TGF-β signaling pathway, angiogenesis, and ROS production (Fig. 2A). Furthermore, we analyzed the relationships between the degrees of liver fibrosis and the above pathways based on the *ssGSEA* scores, and found that hypoxia and ROS pathways stood out (Fig. 2B–F). Liver fibrosis can cause in hypoperfusion of the hepatic lobules, closely related to decreased portal circulation, which ultimately results in intrahepatic hypoxia[27]. And hypoxia, which contributes to the poor prognosis of HCC patients, can increase the expression of hypoxia-inducible factors and activated downstream receptors, leading to the activation of HSCs, abnormal angiogenesis, EMT, and chronic inflammation, especially in advanced fibrosis[27–29]. Activation of HSCs plays an important role in the development of cirrhosis, and can be triggered by numerous signaling molecules, such as ROS and TGF-β[30–33]. In addition, TGF-β is involved in inducing EMT, which plays a crucial role in the progression of cirrhosis and metastasis in HCC patients[34]. Therefore, the above pathways were closely related to occurrence and progression of liver fibrosis, eventually leading to related complications including HCC.

Identification of gene modules related to cirrhosis in the TCGA-LIHC cohort

To identify genes related to the initiation and development of cirrhosis, co-expressed gene networks were constructed using the WGCNA method, and the relationship between each gene module and cirrhosis severity was examined via *Pearson* correlation analysis in the TCGA-LIHC cohort (Fig. 3A–C). A total of 10 gene modules were identified, namely, MEblue, MEturquoise, MEred, MEbrown, MEyellow, MEgreen, MEpink, MEblack, MEmagenta, and MEgray (The genes of each gene module were shown in Table. S2). We found that regardless of the no fibrosis group, MEbrown and MEgreen exhibited the strongest and most significant correlation with portal fibrosis and fibrous septa, respectively ($P < 0.05$, Fig. 3D). Further analyses showed that genes in MEbrown were primarily involved in G-protein coupled receptor signaling pathway and several immune-related functions, including T-cell activation, B-cell receptor signaling, neutrophil and monocyte chemotaxis, macrophage differentiation, and cytokine-mediated signaling (Fig. 3E). In addition, genes in MEgreen were primarily associated with ribosome assembly and translation (Fig. 3F). The above pathways or biological process may be involved in the progression of liver fibrosis, which the genes in MEbrown and MEgreen may play a potential role in.

Enrichment degree of cirrhosis-related gene modules in different cell types

The scRNA-seq data of 10 patients with HCC were extracted from the GSE149614 dataset to identify cell types involved in the occurrence and development of cirrhosis. After filtration, normalization, dimensionality reduction, and clustering, a total of 34,015 cells were selected and categorized into 9 subpopulations (Fig. 4A, S1). Based on the expression of marker genes (Table. S3), the 9 cell subpopulations were identified as follows: hepatocytes ($APOA2^+APOC3^+AHSG^+TTR^+$), macrophages ($C1QA^+FCER1G^+AIF1^+$), NK/T cells ($NKG7^+CD3D^+$), plasma B cells ($IGHG1^+MZB1^+$), proliferative hepatocytes ($TOP2A^+MKI67^+$), endothelial cells ($PLVAP^+CLDN5^+$), myofibroblasts ($ACTA2^+TAGLN^+COL1A1^+LUM^+$), epithelial cells ($EPCAM^+KRT19^+$), and B cells ($MS4A1^+LY9^+$) (Fig. 4B).

The results of the previous analyses suggested that the genes in MEbrown and MEgreen may be involved in liver fibrosis. We further used the *AUCell* algorithm to evaluate the enrichment degree of genes in MEbrown and MEgreen at the above single-cell levels to explore key cell types. Notably, the scores of MEbrown showed higher enrichment in macrophages, NK/T cells, B cells, and plasma cells (Fig. 4C), whereas the scores of MEgreen showed higher enrichment in hepatocytes, epithelial cells, and proliferative hepatocytes (Fig. 4D). These results suggested that the genes in MEbrown and MEgreen genes may play an important role in the development of cirrhosis, which may be closely related to the above immune cells or abnormal proliferative hepatocytes (malignant hepatocytes).

Molecular mechanism of immune cells involved in liver fibrosis

To further investigate the mechanisms through which the immune cells contribute to the development of cirrhosis in HCC, we used the *CellChat* package to identify ligand-receptor pairs in myofibroblasts and main immune cells, including macrophages, NK/T cells, plasma B cells, and B cells, based on the above scRNA-seq data. Several signaling pathways, including TGF-β, fibroblast growth factor (FGF), NOTCH, WNT, EGF, bone morphogenetic protein (BMP) and hepatocyte growth factor (HGF), have be confirmed to be involved in liver fibrosis. For example, TGF-β is widely considered as a crucial mediator in tissue fibrosis[35], and WNT, FGF and NOTCH pathways can play an important role in cell proliferation, differentiation and tissue remodeling[36–38]. Initially, we evaluated the probability of several key signaling pathways involving main ligands-receptor pairs among different cell types (Fig. 5A). The results revealed that there were significant differences in the key pathways of enrichment in different cell types, including several signaling pathways associated with the development of fibrosis, such as TGF-β, FGF, NOTCH, EGF, BMP, and HGF signaling pathways (Fig. 5B–G).

Because ligand-receptor pairs are the key links of signaling pathway transmission, we further analyzed the communication between different cells in HCC based on several key ligand-receptor pairs of TGF-β, FGF, NOTCH, EGF, BMP, and HGF signaling pathways. In particular, we found that the interaction between macrophages and myofibroblasts was the strongest through the above signaling pathways, such as the TGFB1-(TGFBR1 + TGFBR2) pair in the TCG-β signaling pathway, the FGF7-FGFR1 and FGF7-FGFR2 pairs in the FGF signaling pathway, the JAG1-NOTCH3 pair in the NOTCH signaling pathway, the BMP2-(BMPR1B + BMPR2) and BMP2-(BMPR1B + ACVR2B) pairs in the BMP signaling pathway, the AREG-EGFR pair in the EGF signaling pathway, and the HGF-MET pair in the HGF signaling pathway (Fig. 5B–G).

Molecular mechanisms of hepatocytes involved in liver fibrosis

HCC is characterized by high heterogeneity, and different types of cells, including hepatocytes, proliferative hepatocytes and epithelioid cells, may have malignant characteristics in HCC tissues[39]. CNV is one of the most prominent features of tumor cells, so the study chose to analyze these cell types from the perspective of genomic variation to reveal their malignant characteristics[40]. The *inferCNV* package was used to analyze the CNVs profiles of hepatocytes, proliferative hepatocytes, and epithelial cells, with B cells serving as a reference. The results demonstrated that numerous genomic regions in these three cell types were notably amplified or deleted, based on the above scRNA-seq data (Fig. S2). The above results revealed all hepatocytes, proliferative hepatocytes and epithelial cells in primary HCC tissues were malignant in the samples included in this study.

To further explore the mechanisms through which these malignant cells contributed to cirrhosis, we used the *CellChat* package to identify ligand-receptor interactions between myofibroblasts and hepatocytes, proliferative hepatocytes, or epithelial cells. Figure 6A shows the probability of key signaling pathways involving main ligands-receptor pairs in different malignant cell types. Like immune cells, there were significant differences in the key pathways of enrichment in different malignant cell types. including several important signaling pathways

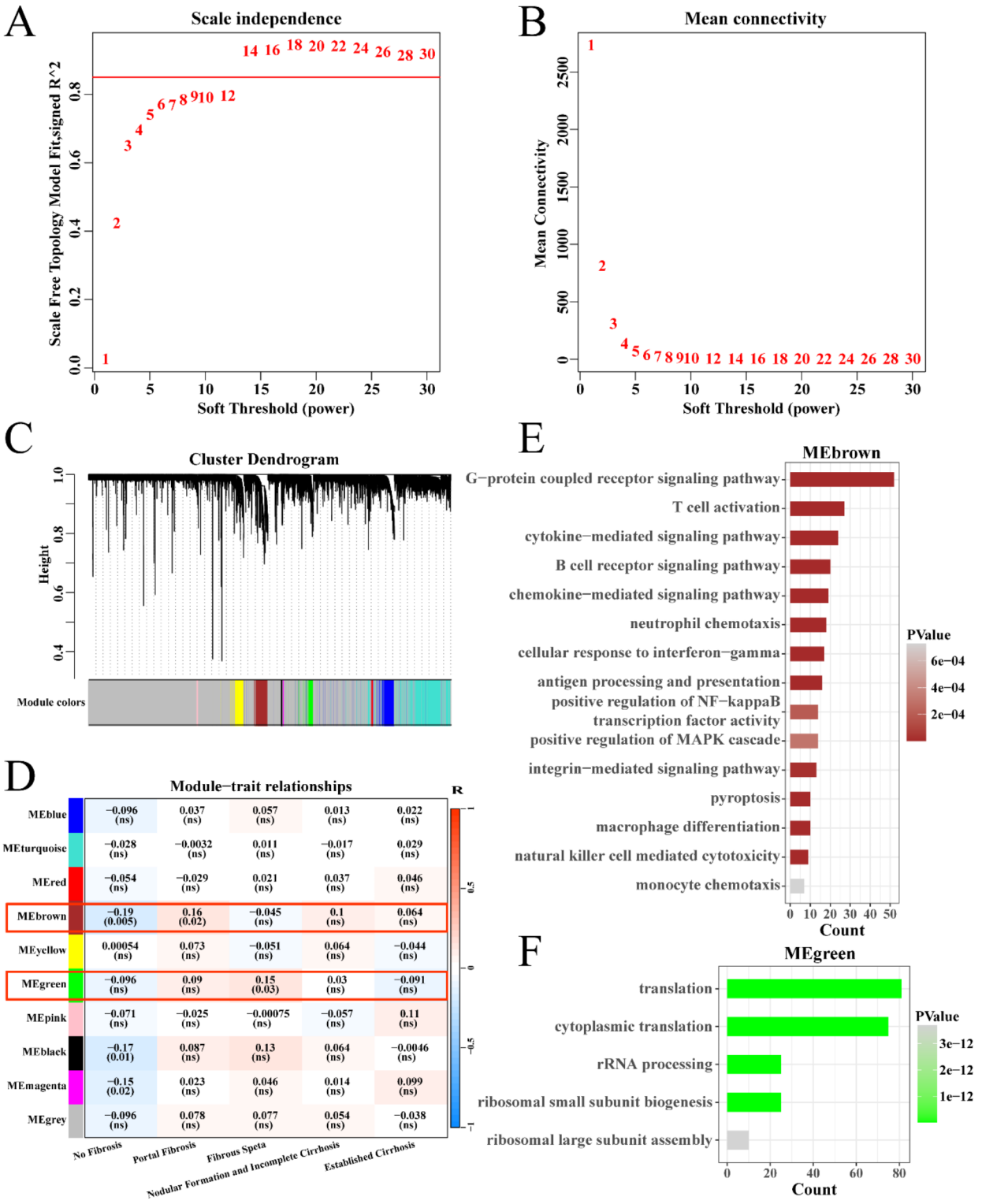

Fig. 3. Identification of cirrhosis-related gene modules in the TCGA-LIHC cohort. (**A**, **B**) Determination of the soft threshold and the relationship between soft threshold and connectivity. (**C**) Gene dendrogram and modules: each leaf represents a gene, whereas each branch represents a co-expression module. (**D**) Correlation between different cirrhosis degrees and gene modules. (**E**, **F**) Enrichment of MEbrown (**E**) and MEgreen (**F**) in biological processes.

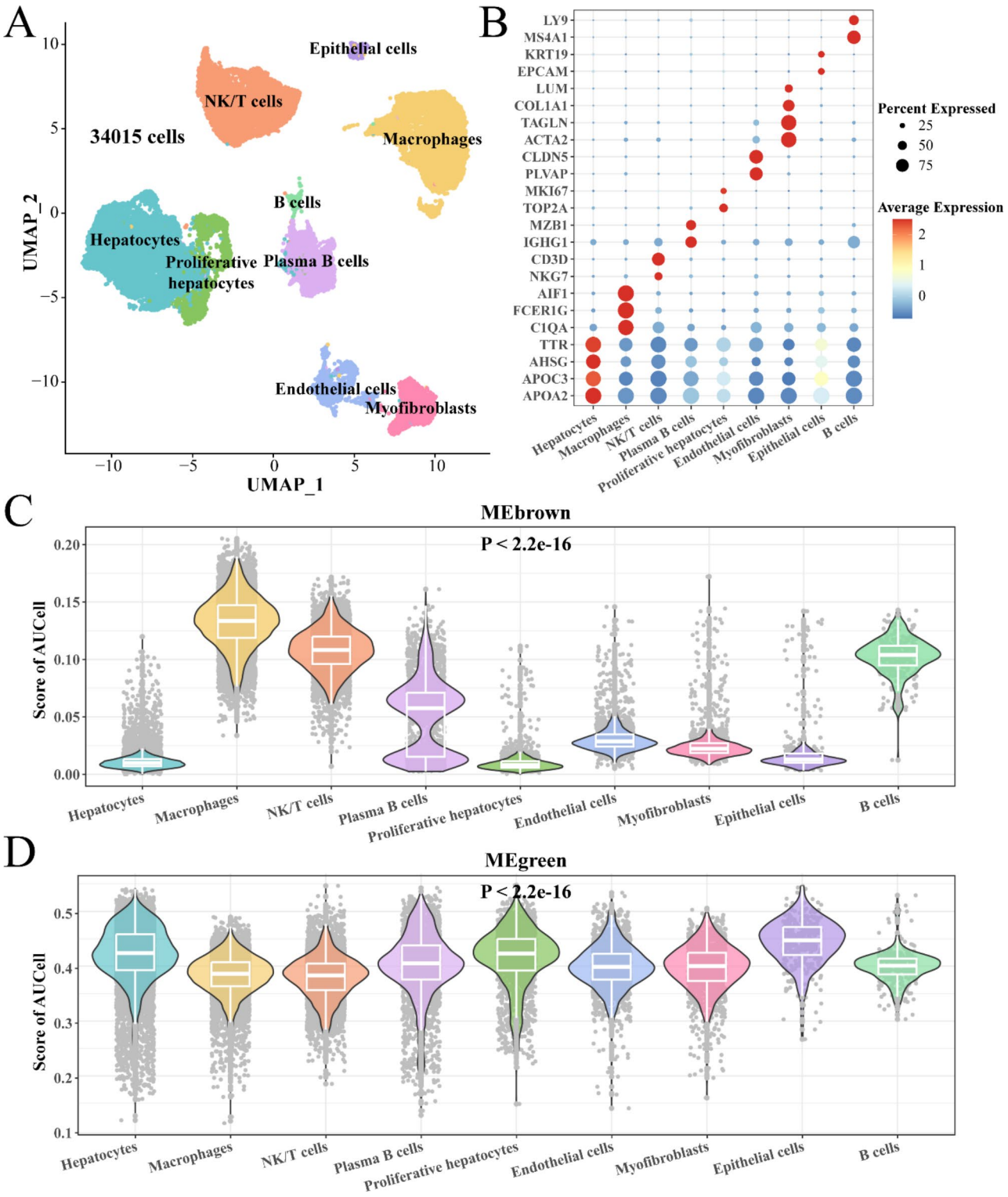

Fig. 4. Enrichment levels of cirrhosis-related gene modules in different cell types based on scRNA-seq. (**A**) Single-cell profiles of patients with primary HCC in the GSE149614 dataset. (**B**) Bubble plot demonstrating the expression of marker genes in each cell type. (**C**, **D**) AUCell enrichment scores of MEbrown (**C**) and MEgreen (**D**) in each cell type.

associated with the development of fibrosis (Fig. 6A), such as TGF-β, FGF, vascular endothelial growth factor (VEGF), WNT, NOTCH, and BMP signaling pathways (Fig. 6B–G).

Specifically, we found that the interactions between hepatocytes and myofibroblasts, and proliferative hepatocytes and myofibroblasts were the strongest through the above signaling pathways, such as the TGFB1-

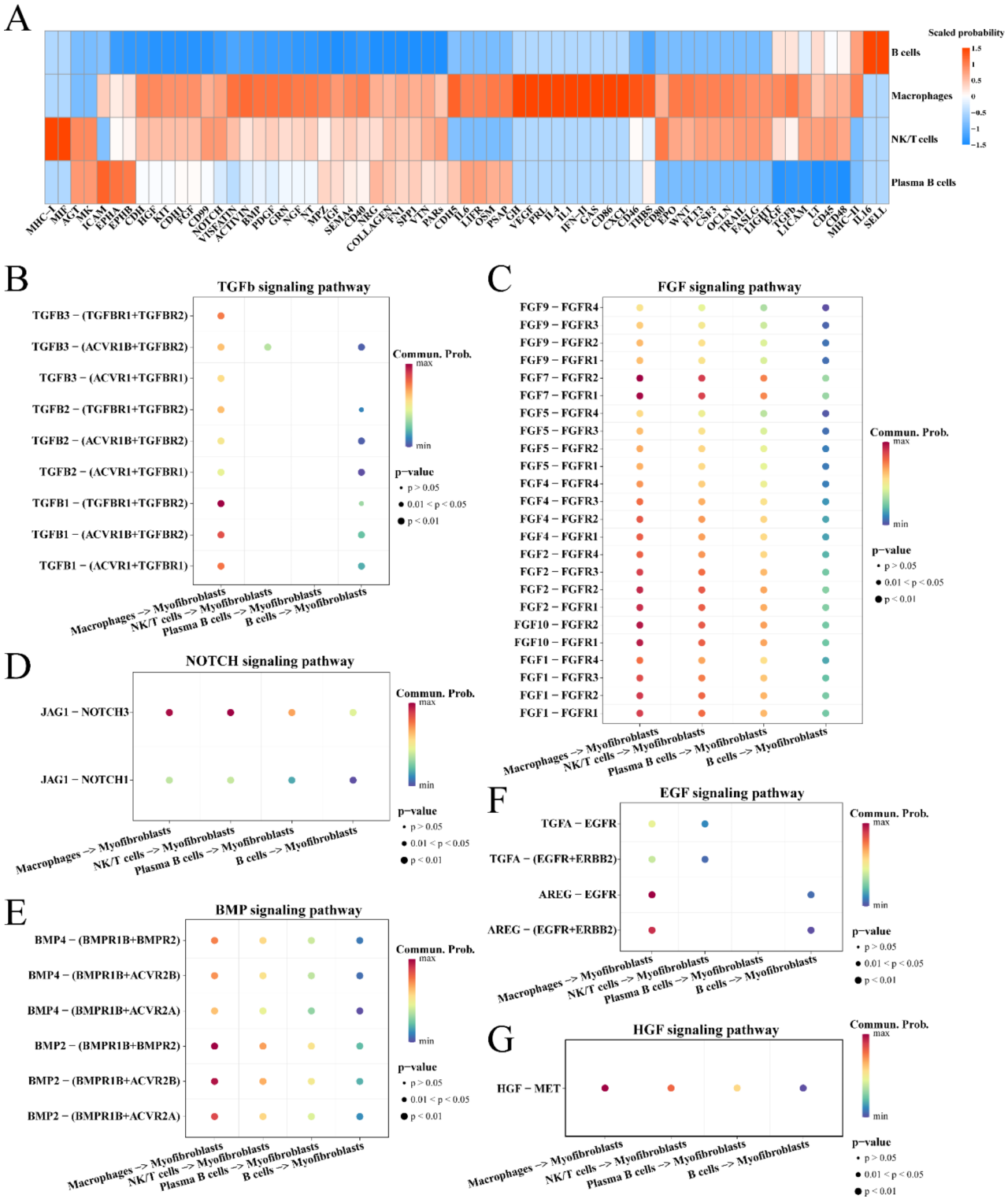

Fig. 5. Analysis of the molecular mechanisms of immune cells involved in fibrosis in HCC. (**A**) The possibility of different immune cell types acting on the singling pathway where the ligand-receptor pairs of myofibroblasts reside. (**B**–**G**) Ligand-receptor pairs in the TGF-β (**B**), FGF (**C**), NOTCH (**D**), BMP (**E**), EGF (**F**), and HGF (**G**) signaling pathway.

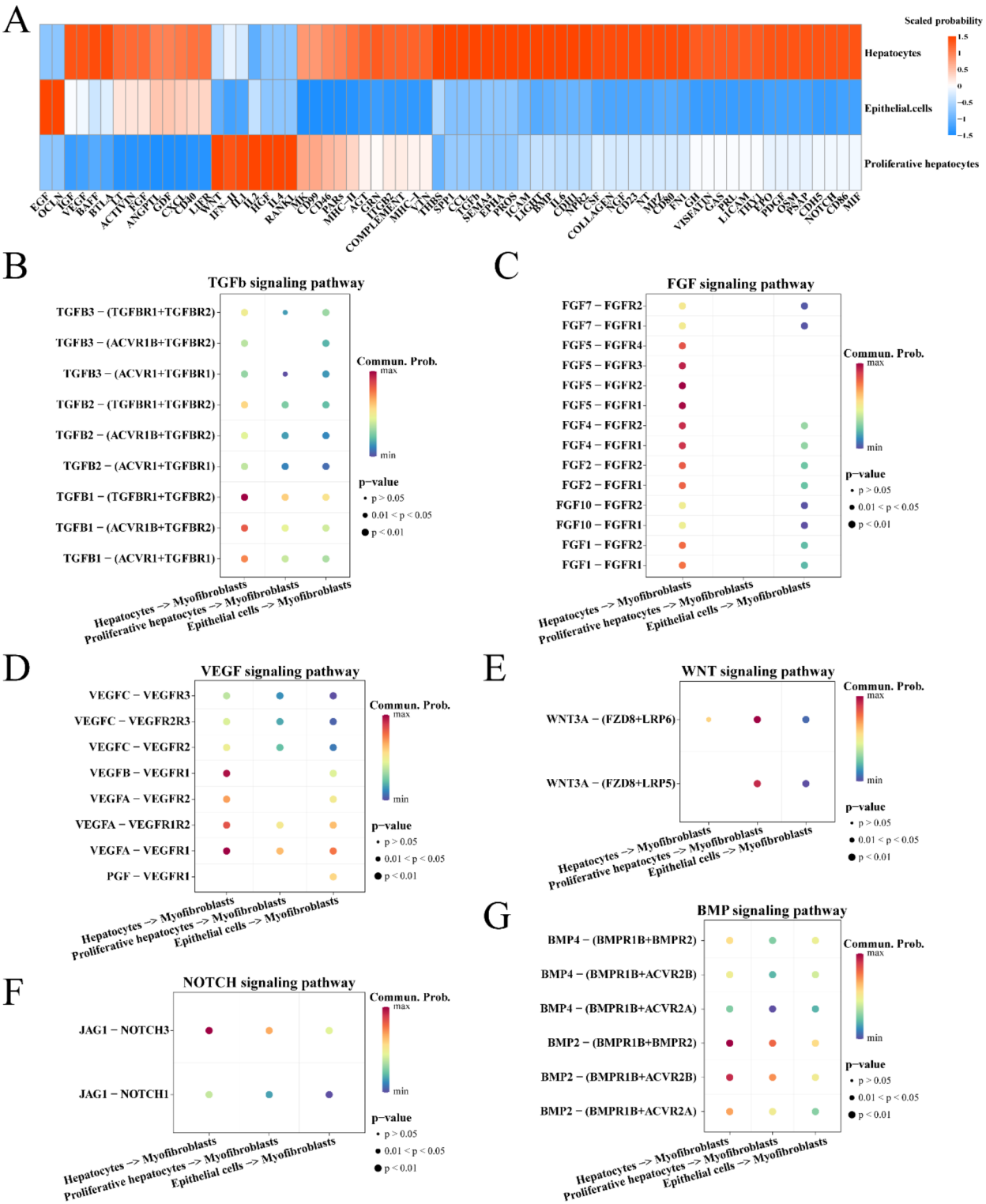

Fig. 6. Molecular mechanisms of malignant cells involved in fibrosis in HCC. (**A**) The possibility of different malignant cells acting on the singling pathway where the ligand-receptor pairs of myofibroblasts reside. (**B**–**G**) Ligand–receptor pairs in the TGF-β (**B**), FGF (**C**), VEGF (**D**), WNT (**E**), NOTCH (**F**), and BMP (**G**) signaling pathway.

(TGFBR1 + TGFBR2) pair in the TCG-β signaling pathway, the FGF5-FGFR2 and FGF5-FGFR1 pairs in the FGF signaling pathway, the VEGFB-VEGR1 and VEGFA-VEGFR1 pairs in the VEGF signaling pathway, the JAG1-NOTCH3 pair in the NOTCH signaling pathway and the BMP2-(BMPR1B + BMPR2) pair in the BMP signaling pathway between hepatocytes and myofibroblasts, and the WNT3A-(FZD8 + LRP6) pair in the WNT signaling pathway between proliferative hepatocytes and myofibroblasts (Fig. 6B–G).

Spatial distribution of different cell types in HCC tissues

The spatial transcriptomic data of patients with HCC were extracted from the GSM7021870 dataset to explore the spatial distribution of different cell types in HCC tissues, providing a reference for further exploring the mechanism of liver fibrosis (Fig. 7A–F, S3). The results indicated that hepatocytes constituted the majority of cells within HCC tissues, with hepatocytes and myofibroblasts being closest to each other (Fig. 7A, B). Additionally, plasma B cells, macrophages and proliferative hepatocytes were in proximity to myofibroblasts (Fig. 7A, C–E). These results suggested that hepatocytes, proliferative hepatocytes, macrophages, and plasma B cells may be spatially close to myofibroblasts and potentially participate in the process of stimulating myofibroblast fibrotic activity, which may be related to the development of liver fibrosis.

Discussion

There are complex intercellular signaling networks between tumors and tumor-associated myofibroblasts[41–43]. The secretion of PDGF and TGF-β by tumors promotes the activation of myofibroblasts, resulting in the formation and progression of fibrosis[9]. In contrast to most malignant tumors, HCC is strongly associated with liver fibrosis[44]. Liver fibrosis promotes tumorigenesis in HCC, whereas fibrosis manifests as reactive desmoplasia after tumor formation in other cancers. Affo et al.[45] suggested that the pre-malignant microenvironment (PME) and TME of HCC should be distinguished. PME, which is formed during the initiation of tumorigenesis, is characterized by persistent liver damage, chronic inflammation, and fibrosis, with fibrosis representing the most distinctive characteristic of hepatic PME. In the healthy liver, HSCs are inactive and are located around sinusoids, characterized by star-shaped morphological features and abundant cytoplasmic lipid droplets[46]. When the liver is injured, activated HSCs differentiate into myofibroblasts that secrete excessive ECM components, pro-inflammatory mediators, α-SMA, and tissue inhibitor of metalloproteinase 1 (TIMP 1)[47–49]. TME consists of diverse components including malignant cells, immune cells, and fibroblasts, all of which significantly influence tumor survival and progression[50]. Given that both immune and malignant cells are involved in the regulation of fibrosis, these cells can be targeted for effective treatment of liver cancer, thus improving the prognosis[51–53]. However, studies investigating the pathological mechanisms of HCC with cirrhosis are limited at present.

In this study, we analyzed the gene expression profiles and clinical characteristics of patients with HCC with cirrhosis in the TIGC-LIHC cohort. The enrichment levels of various cancer-related signaling pathways were evaluated based on the severity of liver fibrosis. The results suggest that plasma albumin levels serve as a promising indicator of the severity of cirrhosis. Administering human albumin transfusions to manage various cirrhosis-associated complications has been shown to provide substantial advantages[54]. Furthermore, the ssGSEA algorithm was utilized to calculated the enrichment scores of 50 hallmarker genes in the TCGA-HILC cohort with cirrhosis. The nodular-formation-and-incomplete-cirrhosis group had the highest enrichment level of five signaling pathways, namely, hypoxia, EMT, TGF-β, angiogenesis, and ROS. Hypoxia can induce excessive production of ROS by the increased generation of free radical species[55]. Additionally, it triggers the activation of the TGF-β signaling pathway and induces angiogenesis and EMT[56]. The TGF-β signaling pathway is considered the strongest stimulator of myofibroblast differentiation[57]. It plays a crucial role in regulating ECM remodeling by promoting the activation of myofibroblasts and accumulation of ECM[58]. EMT is primarily regulated by external signals, with hypoxia and the TGF-β signaling pathway serving as important regulatory factors[59]. Chen et al.[60] showed that induction of EMT promoted the progression and development of liver fibrosis to cirrhosis through the ROS/TGF-β1/Snail-1 signaling pathway. Therefore, we speculate that the five signaling pathways identified in this study participate in the onset and development of liver fibrosis, with hypoxia serving as the primary trigger and orchestrating the other four pathways.

WGCNA revealed 10 gene modules associated with cirrhosis. The AUCell algorithm showed that genes in the MEbrown and MEgreen modules were highly enriched in immune and malignant cells, respectively. Subsequently, the CellChat package and the spatial transcriptomic data of patients with HCC from the GEO dataset were used to validate the relationship between cirrhosis and immune cells or malignant cells. The results indicated that both immune and malignant cells may play a potential role in the pathogenesis of HCC with cirrhosis.

Numerous studies have suggested that reducing macrophage infiltration inhibits the activation of myofibroblasts and alleviates liver fibrosis, highlighting the importance of macrophage–myofibroblast interactions in the progression of fibrosis[61,62]. TGF-β produced by macrophages interacts with corresponding receptors on myofibroblasts, thereby facilitating fibrosis[63]. Yang et al.[64] demonstrated that JAG1 expressed by macrophages interacted with Notch1 on myofibroblasts, promoting NOTCH-mediated activation of HSCs and fibrosis. Li et al.[65] showed that FGF12, which is highly expressed by macrophages, facilitated the pro-inflammatory activation of macrophages and subsequently triggered HSC activation. This study revealed that multiple types of immune cells, in addition to macrophages, and malignant cells can interact with myofibroblasts through diverse signaling pathways, such as TGF-β and NOTCH. Additionally, the catalytic function of EGFR in hepatocytes may be involved in the regulation of fibrosis[66]. We observed that the intercellular communication between malignant cells and myofibroblasts was enriched in a diverse array of signaling pathways, with a particular emphasis on TGF pathway. A previous study, conducted in the 3D biomimetic tumor microenvironment of HCC, revealed that malignant cells stimulated an increase in the expression of TGF-β, which subsequently facilitated the activation and differentiation of HSC[13].

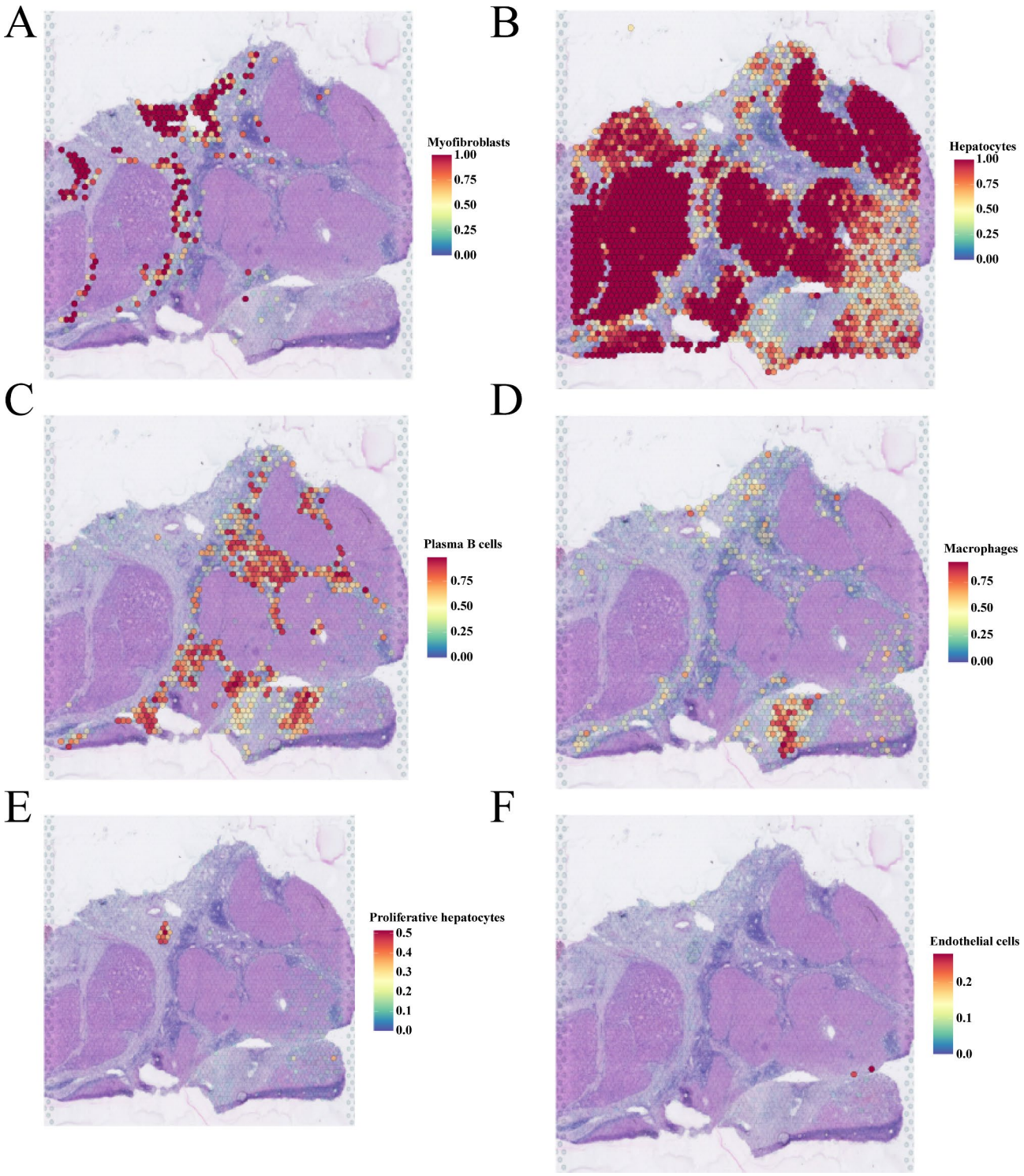

Fig. 7. Spatial distribution of different cell types in HCC tissues. (**A**) Distribution of myofibroblasts in HCC tissues. (**B**–**F**) Distribution of hepatocytes (**B**), plasma B cells (**C**), macrophages (**D**), proliferative hepatocytes (**E**), and endothelial cells (**F**) in HCC tissues.

scRNA-seq and spatial transcriptomic analysis are useful tools for investigating the pathogenesis of HCC with cirrhosis, as they provide in-depth insights into molecular mechanisms at the single-cell level. scRNA-seq enables the analysis of communication and interactions between cancerous and non-cancerous cells in tumors, providing comprehensive insights into various TMEs[67]. Spatial transcriptomic analysis enables the assessment of the spatial distribution of genes at the transcriptional level, providing valuable information regarding tissue characteristics that cannot be obtained through scRNA-seq[68,69]. In this study, the Seurat package was used to

verify cell–cell interactions in HCC tissue and examine the spatial distribution of myofibroblasts, immune cells, and malignant cells. Notably, analysis of scRNA-seq and spatial transcriptomic data suggested strong interactions between myofibroblasts and immune cells as well as between myofibroblasts and malignant cells. Previous studies have shown that myofibroblasts interact with immune or malignant cells through various signaling molecules, thereby affecting tumor survival and growth[4,70]. Therefore, the results of this study indicated that communication between myofibroblasts and immune cells as well as between myofibroblasts and malignant cells may be closely related to the occurrence and development of HCC with cirrhosis.

Although our study offers a novel viewpoint on the molecular mechanisms underlying HCC with cirrhosis, several limitations remain that necessitate further consideration. The analysis presented in this study relies on public databases, thus necessitating clinical cohorts to validate our findings. Additionally, our investigation did not conduct a comprehensive investigation into the intercellular communications and spatial organization among immune cells, malignant cells, and fibroblasts at both the cellular and animal levels. In future work, we intend to enhance the accuracy of our study by conducting analyses of clinical samples and performing experimental validations.

Conclusion

In this study, multi-omic data from the TCGA-LIHC cohort were comprehensively analyzed using various bioinformatic tools. The clinical characteristics and hallmarks of HCC with cirrhosis were preliminarily identified. Additionally, the potential mechanisms through which immune and malignant cells contribute to the development of HCC with cirrhosis were investigated. This study can improve the understanding of the pathological mechanisms underlying the development of HCC with cirrhosis, and provides novel potential strategy for the management of HCC patients.

Data availability

The datasets used and/or analyzed during the current study are available from the corresponding author upon request. In this study, RNA-Seq data from TCGA-LIHC cohort and corresponding clinical characteristics were obtained from the UCSC Xena database (https://xena.ucsc.edu/). 50 hallmarker genes and their related gene symbols were extracted from MsigDB (https://www.gsea-msigdb.org/gsea/msigdb). The GSE14961 and GSM7021870 datasets were downloaded from the Gene Expression Omnibus (https://www.ncbi.nlm.nih.gov/geo/).

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Declarations

Competing interests
The authors declare no competing interests.

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