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Screening for lipid metabolism-related genes and identifying the therapeutic potential of ACACA for ER stress-related progression in hepatocellular carcinoma

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How to cite this article: Liu X, Li S, Rao P, Yu W, Tang Y, Wang A, Chen M, Sun G, Liu J. Screening for lipid metabolism-related genes and identifying the therapeutic potential of ACACA for ER stress-related progression in hepatocellular carcinoma. *Hepatoma Res.* 2025;11:13. <https://dx.doi.org/10.20517/2394-5079.2024.135>

Received: 24 Nov 2024 **First Decision:** 15 Apr 2025 **Revised:** 27 Apr 2025 **Accepted:** 26 May 2025 **Published:** 29 May 2025

Academic Editor: Xiangmei Chen **Copy Editor:** Ting-Ting Hu **Production Editor:** Ting-Ting Hu

Abstract

Aim: The reprogramming of lipid metabolism can markedly enhance the nutritional adaptability of tumor cells to the glucose-deficient and hypoxic tumor microenvironment, which holds profound significance for the development and metastasis of liver cancer. Nevertheless, the alterations of lipid metabolism under stress conditions and the specific mechanisms remain ambiguous. The current study aimed to explore the molecular interaction between endoplasmic reticulum (ER) stress and lipid metabolism in hepatocellular carcinoma (HCC) using bioinformatics analysis, and further verify the role of core hub genes and offer potential targets for diagnosing and treating HCC.

Methods: Differentially expressed lipid-related genes (DLRGs) were identified via cross-crossing differentially expressed genes (DEGs) in the TCGA-LIHC program and lipid metabolism-related genes in the Genecards database. Identification of hub genes was achieved by constructing a protein-protein interaction (PPI) network,



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gene ontology (GO), and Kyoto Encyclopedia of Genes and Genomes (KEGG). Disease correlation analysis was performed in the Comparison of Toxicology Database, receiver operating characteristic (ROC) curve and Kaplan-Meier curve analyses were performed for the hub genes, and the CIBERSORT algorithm was employed to assess immune infiltration. The role of acetyl-CoA carboxylase 1 (ACACA) in HCC was evaluated by Western blotting, polymerase chain reaction, immunohistochemistry, and CCK-8 assay.

Results: In total, 131 DLRGs were identified, comprising 70 upregulated and 61 downregulated. PPI analysis identified 20 hub DLRGs, while ROC curve analysis and Kaplan-Meier analysis further revealed that ACACA, LCAT, APOC3, LPA and PON1 may hold diagnostic and prognostic value for HCC patients. More importantly, ACACA overexpression was related to unfavorable overall survival (OS) and adverse pathological characteristics in HCC patients. In addition, both free fatty acid (FFA) and tunicamycin (TM) could activate ER stress and enhance the expression of ACACA in HCC. Interestingly, inhibition of ER stress or fatty acid synthesis using 4-phenylbutyric acid (4-PBA) or fenofibrate significantly reduced the expression of ACACA, and fenofibrate inhibits HCC cell proliferation.

Conclusion: The study identifies a novel core lipid metabolism-related gene called ACACA, which has prognostic and therapeutic potential for HCC. We also provide a deep understanding of lipid metabolism correlated with ER stress in the progression of HCC, offering new opportunities for the identification of biological targets and the development of drugs and treatments for HCC patients.

Keywords: Hepatocellular carcinoma, lipid metabolism, ACACA, endoplasmic reticulum stress, bioinformatics analysis

INTRODUCTION

Hepatocellular carcinoma (HCC) is a common type of malignancy worldwide, and the prognosis for advanced patients is extremely poor^[1]. Surgery is of crucial significance in the treatment of HCC; however, a large proportion of patients are diagnosed in the late stage. Even among those who undergo timely radical surgery, 20%-25% of them recur within 5 years after surgery^[1]. Systemic chemotherapy is of great importance in the management of advanced HCC; however, the efficacy of current chemotherapeutic agents is limited due to chemotherapy resistance^[2]. Therefore, it is urgent to develop effective therapeutic agents for HCC. The liver, one of the major metabolic organs in the human body, has a key impact on metabolizing nutrients like sugars, lipids, and proteins. Viral hepatitis, alcoholic liver disease, and nonalcoholic fatty liver disease (NAFLD) are common chronic liver diseases closely associated with the incidence of HCC^[3]. Metabolic disorders within the hepatic microenvironment caused by viral infection, chronic inflammation, and the initiation and progression of liver cancer are significantly influenced by metabolic syndrome^[4]. Therefore, targeting the metabolic disorder theoretically has the possibility to achieve new breakthroughs in preventing and treating liver cancer. However, to date, no substantial progress has been achieved in treating liver cancer.

HCC is characterized by complex metabolic reprogramming, especially in lipid metabolism. Studies have identified significant alterations in lipid metabolic pathways that contribute to the initiation and progression of HCC^[5,6], such as fatty acid synthesis, β -oxidation, and the modification of cellular lipid compositions. HCC cells frequently exhibit increased *de novo* lipogenesis (DNL) and modification of fatty acid oxidation pathways. Overexpression of enzymes such as ATP citrate lyase, stearyl-CoA desaturase, and fatty acid synthase (FASN) has been observed in HCC, highlighting the importance of these pathways in tumorigenesis^[7,8]. Acetyl-CoA derived from citric acid (from glucose and glutamine) or exogenous acetic acid is catalyzed by acetyl-CoA carboxylase 1 (ACACA) to produce malonyl-CoA, which is then converted into fatty acids by FASN to promote DNL^[9]. In addition, the hypoxic tumor microenvironment can produce

large amounts of acetic acid, which induces histone acetylation and promotes the expression of FASN and ACACA, thereby facilitating lipid synthesis^[10]. The HCC cells drive lipid metabolic reprogramming mainly by altering the expression of enzymes related to lipid metabolism, but the mechanism by which the stress microenvironment regulates the activity of lipid metabolism enzymes and related molecules remains unclear.

Endoplasmic reticulum (ER) stress is usually triggered owing to the aggregation of misfolded or unfolded proteins within the ER lumen, which, in turn, activates the unfolded protein response (UPR). The UPR functions to revive ER capability by boosting protein folding capacity, degrading misfolded proteins, and diminishing protein synthesis^[11]. Research has illustrated that ER stress interferes with lipid metabolism at several levels. For example, in hepatocytes, ER stress hampers cholesterol efflux and synthesis by reducing the expression and function of ATP-binding cassette transporter A1 (ABCA1) and disrupting the functioning of HMG-CoA reductase^[12]. Recent studies have also shown that activation of UPR sensors can significantly alter lipid metabolic pathways, influencing enzymes that contribute to the synthesis and regulation of fatty acids, triglycerides, phospholipids, and cholesterol^[13]. For example, activating transcription factor 4 (ATF4) and C/EBP-homologous protein (CHOP), downstream effectors of the protein kinase R-like ER kinase (PERK) pathway, are involved in the regulation of fibroblast growth factor 21, a central mediator in lipid metabolism^[14]. A key aspect of ER stress and lipid metabolism is its role in lipotoxicity. Excessive lipid accumulation induces ER stress, which in turn exacerbates lipid dysregulation, creating a vicious cycle that leads to cell death and tissue dysfunction. This phenomenon is particularly evident in peripheral organs such as the liver, muscle, and heart, where lipotoxicity contributes to the pathogenesis of metabolic diseases^[11,15]. ER stress also influences lipid metabolism in macrophages by facilitating cholesterol uptake and hampering cholesterol efflux, further exacerbating atherosclerosis^[16]. These interactions highlight the importance of maintaining ER homeostasis for proper lipid metabolic function and suggest potential targets for therapeutic intervention in metabolic diseases.

In this investigation, we aimed to explore the molecular interactions between ER stress and lipid metabolism in HCC using bioinformatics technology, and further validate the core hub gene in the development of HCC. Therefore, we surveyed the differentially expressed genes (DEGs) of lipid metabolism using TCGA-LIHC projects and Genecards databases. Then, the molecular mechanisms of identified DEGs in the development of HCC and its potential prognostic efficacy were measured by constructing the protein-protein interaction (PPI) network, Gene Ontology (GO), and the Kyoto Encyclopedia of Genes and Genomes (KEGG). Moreover, the role of the identified hub gene in ER stress-related lipid metabolism was further verified according to several *in vitro* experiments. Our work provides a theoretical underpinning and potential target for treating HCC by reprogramming lipid metabolism.

METHODS

Data collection

Lipid metabolism-related genes (LRGs) were obtained from the Genecards database (<https://www.genecards.org/>) with a minimum relevance score of no less than 5.0, and a total of 701 LRGs were obtained. The HCC dataset was sourced from TCGA (<https://portal.gdc.cancer.gov/>). The HCC dataset encompassed 374 cases of liver cancer samples and 50 cases of liver tissue samples, and all the samples were accompanied by clinical data. Venn diagrams were plotted using an online tool (<http://www.ehbio.com/test/venn/#/>) and common DEGs were calculated from the intersection of the LRGs datasets and DEGs in the TCGA-LIHC database.

Identification of DEGs

The TCGA program for HCC was employed to screen and identify the DEGs. An adjusted P -value < 0.05 and $\log_{2}FC > 1.0$ were adopted as the cutoff criteria, where the DEGs with $|\log_{2}FC| > 1.0$ were classified as genes highly expressed, while the DEGs with $\log_{2}FC < -1$ were deemed as genes with decreased expression. Additionally, differentially expressed lipid-related genes (DLRGs) were defined as the DEGs from the TCGA dataset that overlapped with the LRGs in the Genecards database using the Venn diagram, and HemI 2.0 was exploited to draw the heat map of the commonly expressed DEGs. Packages such as Complex Heatmap, ggplot2 [3.3.6], and other R packages were utilized for generating heat maps and volcanic maps of the identified DEGs.

GO and KEGG analysis

The online version 6.8.0 of the Database for Annotation, Visualization and Integrated Discovery (DAVID, <https://david.ncifcrf.gov/home.jsp>) was applied for GO annotation and KEGG pathway analysis, and the threshold was set at $P < 0.05$ and counts ≥ 2 , whereas the top 10 were selected based on a P -value < 0.01 . Moreover, biological process (BP), molecular function (MF), and cellular component (CC) were integrated into the analysis of GO terms. The R's ggplot2 (version 3.3.2) software was employed to display the analysis results.

PPI network

The STRING database (version 11.0, <https://string-db.org/>) was exploited to structure the PPI network and to elucidate the relationships among proteins encoded by the previously screened DLRGs. The minimum score for interaction was set at 0.7. Furthermore, the results obtained from the STRING online database were then incorporated into the Cytoscape (version 3.8.0, <https://cytoscape.org/>) plug-ins for visualizing the molecular interaction networks and screening key DLRGs. The selection of hub genes was based on connection degree using the CytoHubba model, and the top 20 DLRGs were used for further analysis.

The Comparative Toxicomics Database

The Comparative Toxicomics Database (CTD) provides extensive data on the complex interplay between chemical exposures, genes, proteins, and diseases. In this study, we availed of the CTD database to assess the inferred scores of hub genes across various liver diseases.

Drawing the ROC curve

The receiver operating characteristic (ROC) curve can help us determine whether the gene has some degree of diagnostic value. Therefore, we performed a ROC curve analysis of the 20 DLRGs using the R package pROC. The area under the curve (AUC) was employed to define the predictive reliability. The cutoff value was specified as $AUC > 0.5$, and the AUC values of 0.5 to 0.7, 0.7 to 0.9, and beyond 0.9 were classified as low, moderate, and high accuracy, respectively.

Survival analysis

For survival analysis, DLRGs were assigned into low- and high-expression groups according to the expression value using R language. The hazard ratios (HR) were measured using a Cox regression model, and the survival curve was generated based on Kaplan-Meier estimation.

Potential therapeutic agents

Potential therapeutic agents were identified utilizing the online Drug Gene Interaction Database (DGIdb, <https://dgidb.org/>) based on five core DLRGs, and the interaction networks were visualized using the Cytoscape software.

Immune cell infiltration analysis

To evaluate the relationship between core DLRGs and the infiltration of immune cells in HCC tissues, Spearman correlation analysis was carried out using the TCGA-LIHC database to determine whether the 5 core DLRGs are related to immune infiltration using CIBERSORTx databases. The single-sample gene set enrichment analysis (ssGSEA) was employed to examine the connection between the expression levels of 5 core DLRGs and the proportions of 6 immune infiltrates, namely B cells, Cytotoxic cells, CD8⁺ T cells, Neutrophils, Macrophages, and Dendritic cells, leveraging the ggplot2 [3.3.6], stats [4.2.1], and car [3.1-0] R packages. A *P*-value less than 0.05 was deemed to indicate a statistically difference, and non-participants were excluded from the analysis.

Immunohistochemistry assay

Eighty-five cases of human HCC tissues and paired adjacent normal tissues were formalin-fixed and paraffin-embedded to construct tissue microarrays at the First Affiliated Hospital of Anhui Medical University. Tumors were classified according to the WHO classification system and the International Union Against Cancer tumor-node-metastasis (TNM) classification system. Tissues were sectioned into 4 μm slices and arranged for immunohistochemistry (IHC) examination. In brief^[17], the slides were dried in an incubator at 60 °C, dewaxed to water, antigenically repaired, endogenous peroxidase blocked, and blocked with goat serum. Then, the slides underwent incubation with anti-ACACA (21923-1-AP, Proteintech) and Anti-human GRP78 (ab32618, Abcam) at 4 °C throughout the night. Following this, the slides were rinsed and co-incubated with a biotinylated secondary antibody for 60 min at 37 °C and a peroxidase-conjugated streptavidin. Ultimately, the slides were made visible with diaminobenzidine and underwent counterstaining with hematoxylin under an Olympus microscope. The expression levels were scored by multiplying the staining intensity (0 for negative, 1 for weak intensity, 2 for moderate intensity, 3 for strong intensity) with the fraction of stained cells (< 25%, 25%~49%, 50%~75% and > 75% represent 0, 1, 2, and 3, respectively). We defined a high expression staining score as no less than 6 and a low expression score as less than 6.

Cell culture

Human LO-2 hepatocytes and HCC cell lines HepG2 (Cell Bank of the Chinese Academy of Sciences, Shanghai, China) were cultivated in high-glucose Dulbecco Modified Eagle medium (DMEM) containing 10% fetal bovine serum (FBS, Clark, Australia) and 1% penicillin-streptomycin (Life Technologies, CA, USA). All cells were kept at 37 °C in an atmosphere with 5% CO₂.

Cell viability assay

The cell counting kit-8 (CCK-8; Dojindo, Japan) was utilized to assess cell viability. HepG2 cells were placed onto a 96-well plate and co-cultured with varying concentrations of fenofibrate combined with palmitic acid (PA) or relative control for a duration of 24 h. Thereafter, each well received 10 μL of CCK-8 solution, and the absorbance values at 450 nm were evaluated numerically using an enzyme-linked immunosorbent assay meter.

Real-time quantitative PCR

Trizol reagent (Invitrogen, USA) was employed to purify total RNA from HepG2 cells, and the concentration of RNA was measured via the Nanodrop 2,000 spectrophotometer (Thermo Scientific, USA). The complementary DNA (cDNA) was reversely transcribed through real-time quantitative PCR (qRT-PCR) employing HiScript II Q RT SuperMix (Vazyme, Nanjing, China) as per the guidance of the manufacturer. qRT-PCR analyses for *ACACA*, *FASN*, *PPAR-γ*, *C/EBP-β*, and *GAPDH* were executed utilizing the AceQ qPCR SYBR Green Master Mix (Vazyme, Nanjing, China) under standardized conditions. [Supplementary Table 1](#) provides detailed information about the primer sequences.

Western blotting analysis

The expression levels of GRP78, PERK, ATF6, IRE1 α , ACACA, BCL-2, BAX, GAPDH, and β -actin were evaluated through Western blotting analysis. Briefly^[17], cells or tissues were lysed with freshly prepared protein lysis buffer, which consists of RIPA: PMSF in a ratio of 100:1. Quantification of total cellular proteins was carried out via BCA protein quantification kit (Thermo Fisher Scientific). Proteins were first separated by SDS-PAGE gel and then transferred onto PVDF membranes (Millipore, Bedford, MA, USA). The membranes were incubated overnight with primary antibodies, followed by incubation with the appropriate secondary antibodies (diluted 1:10,000) for 2 h at 37 °C. Finally, protein bands were visualized utilizing the Image Quant™ LAS-4000 Mini Developer system (Fuji, Japan), and the intensity was semi-quantitatively analyzed using Scion Image (version 4.0.3.2). The specific antibodies used are cataloged in [Supplementary Table 2](#).

Statistical analysis

Statistical analyses employed SPSS 23 software (SPSS Inc., Chicago, Illinois, USA); each experiment was conducted no less than three times, with the results displayed as the mean plus/minus the standard deviation (SD). A t-test facilitated statistical evaluation for two-group comparisons, whereas one-way ANOVA addressed comparisons of no fewer than three groups. A *P*-value less than 0.05 denoted a statistically significant difference.

RESULTS

Recognition of the DLRGs in HCC

In an effort to identify DLRGs in HCC, we began by acquiring the mRNA sequencing data from the TCGA database containing 374 cases of HCC and 50 normal cases. $|\log FC| > 1$ and $P < 0.05$ were set as the thresholds, and 2,897 genes (including 2,451 overexpressed genes and 446 downregulated genes) were defined as DEGs from TCGA-LIHC [\[Figure 1A\]](#). Subsequently, these DEGs were intersected with 701 LRGs retrieved from the Genecards database with a minimum relevance score of no less than 5.0, and 131 differentially expressed LRGs (DLRGs) were obtained for further analysis [\[Figure 1B\]](#). The heat map was constructed based on the expression levels of the 131 DLRGs from the TCGA-LIHC project [\[Figure 1C\]](#) using the ComplexHeatmap [\[2.13.1\]](#) R package.

GO function and KEGG pathway analysis

To examine the bioactivity and pathways associated with DLRGs in the development of HCC, GO enrichment analysis and KEGG pathway enrichment analysis were performed using the online database DAVID 6.8. Visualizations were performed using the ggplot2 [\[3.3.6\]](#) R package based on the 131 DLRGs. Upon integration with expression levels ($\log FC$), the Z-scores indicated that the DLRGs (Z -scores > 0) had a positive regulatory effect on all the significant terms, and the 131 DLRGs were predominantly enriched in 569 BP terms, 52 CC terms, and 120 MF terms [\[Figure 2A\]](#). GO analysis demonstrated that the DLRGs were mainly concentrated in BP related to lipid localization, steroid metabolic process, and fatty acid metabolic process. The KEGG pathway enrichment analysis, as presented in [Figure 2B](#), indicated that the 131 DLRGs were predominantly linked to the PPAR signaling pathway (17 genes), cholesterol metabolism (14 genes), glycerophospholipid metabolism (14 genes), sphingolipid metabolism (10 genes), and fatty acid metabolism (9 genes). The most significant (top 5) terms of GO and KEGG analysis are presented in [Supplementary Table 3](#).

Assessment of the PPI network and recognition of hub genes

Utilizing the STRING database, the PPI networks of the 131 DLRGs, which encompassed 131 nodes and 755 edges, were examined, and the average node degree was 11.5 between proteins [\[Figure 3A\]](#). Using the CytoHubba model, cell landscape network visualization was obtained based on the STRING database, and

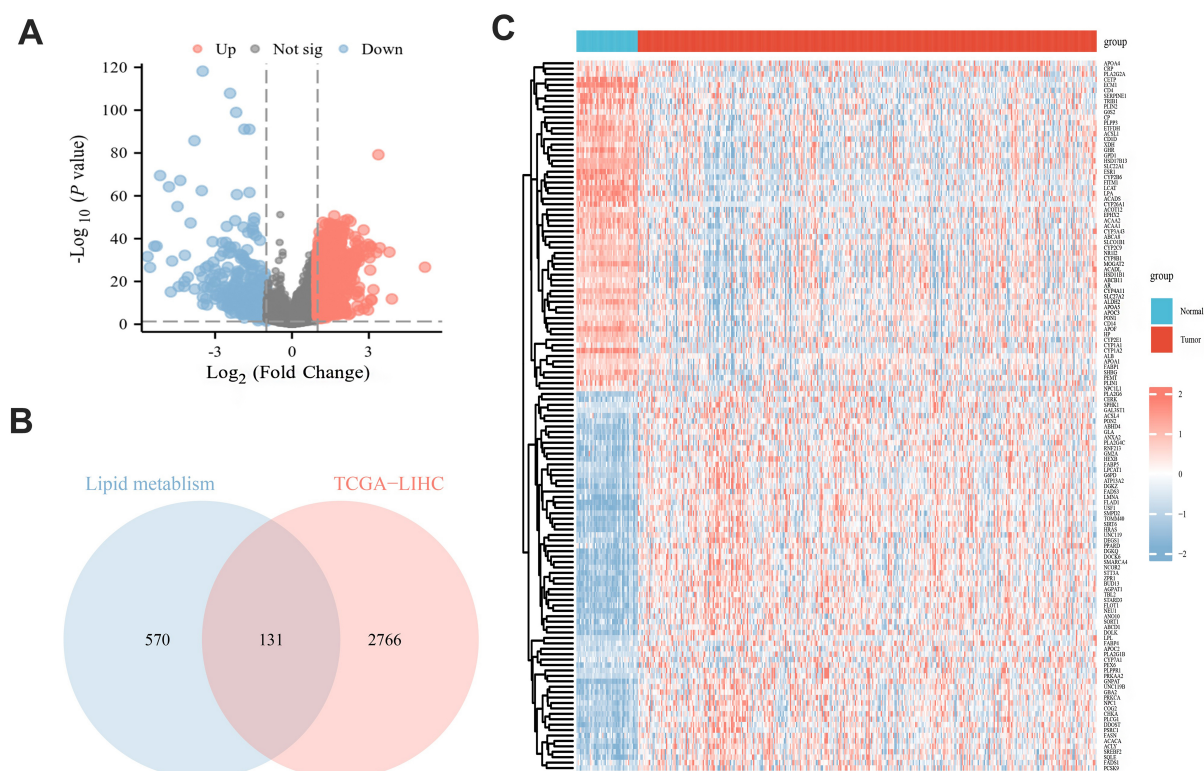


Figure 1. Recognition of the DLRGs. (A) Volcano plot for the differentially expressed genes between normal and HCC groups in TCGA-LIHC database; (B) Venn diagram of LRGs from Genecards and differential expressed genes from TCGA-LIHC; (C) Heatmap of the 131 DLRGs in TCGA-LIHC database. DLRGs: Differentially expressed lipid-related genes; LIHC: Liver Hepatocellular Carcinoma; LRGs: Lipid metabolism-related genes; TCGA: The Cancer Genome Atlas; HCC: hepatocellular carcinoma.

the top 20 central node genes were identified as hub genes ranked by the MCC method. The top 20 hub genes were *LPL*, *APOA1*, *APOC3*, *APOA5*, *LCAT*, *CETP*, *ALB*, *APOC2*, *LPA*, *APOA4*, *PON1*, *APOF*, *PCSK9*, *CYP7A1*, *NPC1L1*, *CRP*, *FASN*, *ACSL1*, *ACACA*, and *ACADL* [Figure 3B], which were defined as the core DLRGs. An examination via metascape analysis of the core DLRGs demonstrated that these DLRGs were predominantly implicated in plasma lipoprotein remodeling, cholesterol metabolism, fatty acid metabolism, response to extracellular stimulus, and the PPAR signaling pathway [Figure 3C].

Evaluation of the correlation between core DLRGs and liver diseases

The expression patterns of the 20 core DLRGs between tumor and non-tumor tissues were analyzed in 374 cases of HCC tissues and 50 cases of non-tumor tissues from the TCGA-LIHC datasets using GEPIA. As shown in Figure 4A, all core DLRGs were significantly differentially expressed between non-tumor and tumor tissues. Among the core DLRGs, the expression levels of *LPL*, *APOC2*, *PCSK9*, *CYP7A1*, *FASN*, and *ACACA* were markedly elevated in HCC tissues compared to non-tumor tissues, whereas the other 14 DLRGs was decreased in HCC tissues. To explore the correlation of these core DLRGs, we performed Spearman correlation analysis for the 20 core DLRGs and found that the expression of most upregulated genes was positively correlated with each other [Figure 4B]. To further estimate the conceptual associations between the forecasted core DLRGs and chemicals or environmental exposure circumstances, we analyzed 20 core DLRGs in the CTD, and 6 liver diseases were selected, including liver neoplasms, liver neoplasms (experimental), fatty liver (alcoholic), liver cirrhosis, liver cirrhosis (experimental), and hepatitis [Figure 4C]. The mean inference scores of the core DLRGs in liver neoplasms and experimental liver neoplasms were 175.09 ± 59.69 and 139.36 ± 33.09 , respectively, which were higher than those in chronic liver diseases (91.8 ± 58.22). These findings suggested that the DLRGs might be involved in various pathophysiological processes in liver diseases.

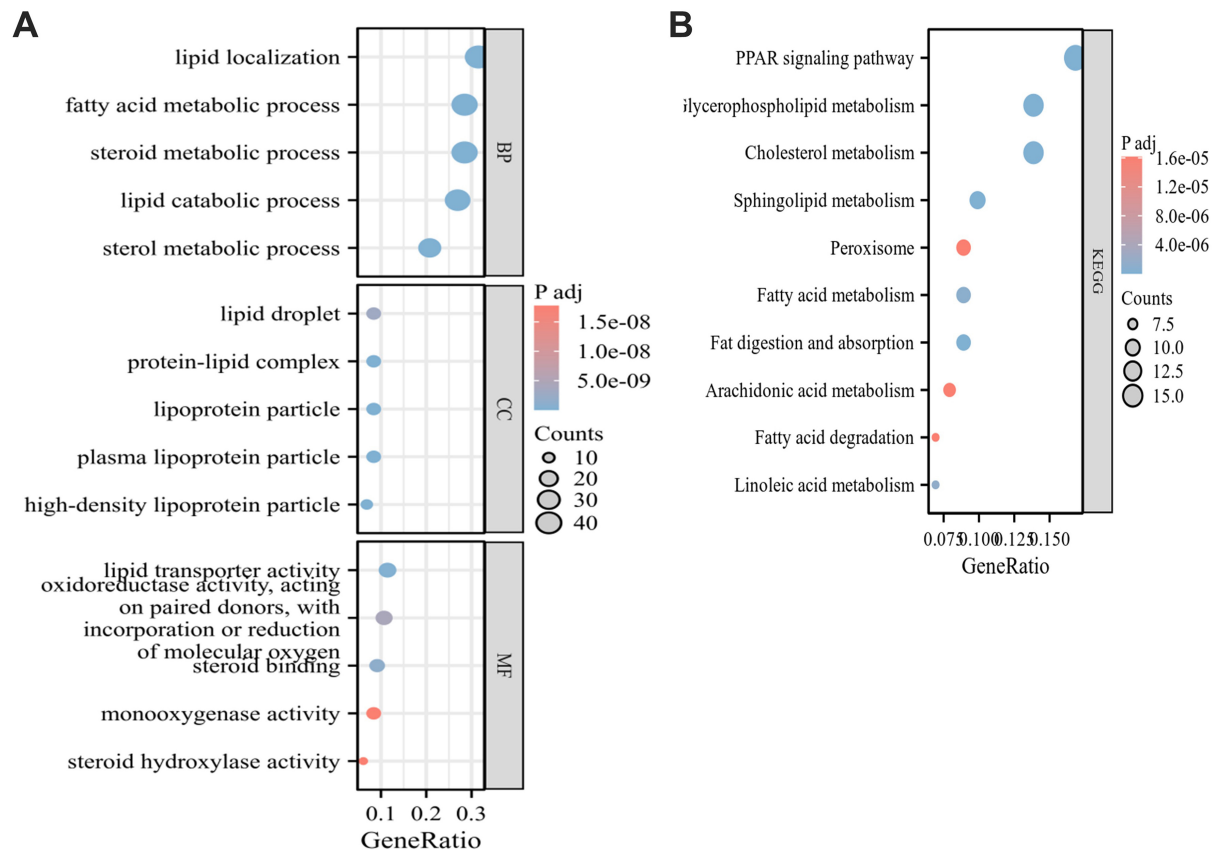


Figure 2. GO and KEGG analyses of DLRGs. (A) GO enrichment analyses of the 131 DLRGs as demonstrated by Bubble plot analysis; (B) KEGG pathway analysis of the 131 DLRGs (top 10). Red denotes upregulated genes, and blue denotes downregulated genes. GO: Gene ontology; KEGG: Kyoto Encyclopedia of Genes and Genomes; DLRGs: differentially expressed lipid-related genes.

Expression levels of core DLRGs in HCC patients and their potential prognostic efficacy

We performed ROC and Kaplan-Meier curves to understand the precise role of core DLRGs in the progression of HCC. ROC curve is frequently used to accurately diagnose diseases based on its sensitivity and specificity. In the current study, a ROC curve was applied to analyze the 20 core DLRGs in HCC and liver tissues, and an AUC above 0.75 was set as the criterion. It was found that the AUC value of 16 core DLRGs was more than 0.75, including *LPL*, *APOA1*, *APOC3*, *APOA5*, *LCAT*, *CETP*, *ALB*, *APOC2*, *LPA*, *PON1*, *APOF*, *PCSK9*, *FASN*, *ACSL1*, *ACACA*, and *ACADL* [Figure 5A]. Therefore, these 16 core DLRGs are eligible for use as diagnostic biomarkers for early diagnosis of liver cancer. The prognostic value of a single core DLRG was assessed by GEPIA to generate survival plots depicting the OS and disease-free survival (DFS). As shown in Figure 5B, HCC patients with higher levels of *ACACA* showed worse OS and DFS than those with lower levels, whereas *LCAT*, *APOC3*, *LPA*, and *PON1* showed an opposite trend. However, *LPL*, *APOA1*, *APOA5*, *CETP*, *ALB*, *APOC2*, *APOF*, *PCSK9*, *FASN*, *ACSL1*, and *ACADL* were not correlated with OS and/or DFS. These results suggested that *ACACA*, *LCAT*, *APOC3*, *LPA*, and *PON1* may have both diagnostic and prognostic values in HCC patients and were selected for further analysis. Moreover, we further explored the relationship between clinical features (such as pathologic stage, tumor status, histological type, histologic grade, vascular invasion, and adjacent hepatic tissue inflammation) and the 5 core DLRGs (*ACACA*, *LCAT*, *APOC3*, *LPA*, and *PON1*) using correlation heatmap analysis and noted that the level of *ACACA* was favorably related to malignant biological behavior, such as a higher pathologic stage, worse tumor status, lower histologic grade, etc. [Figure 5C].

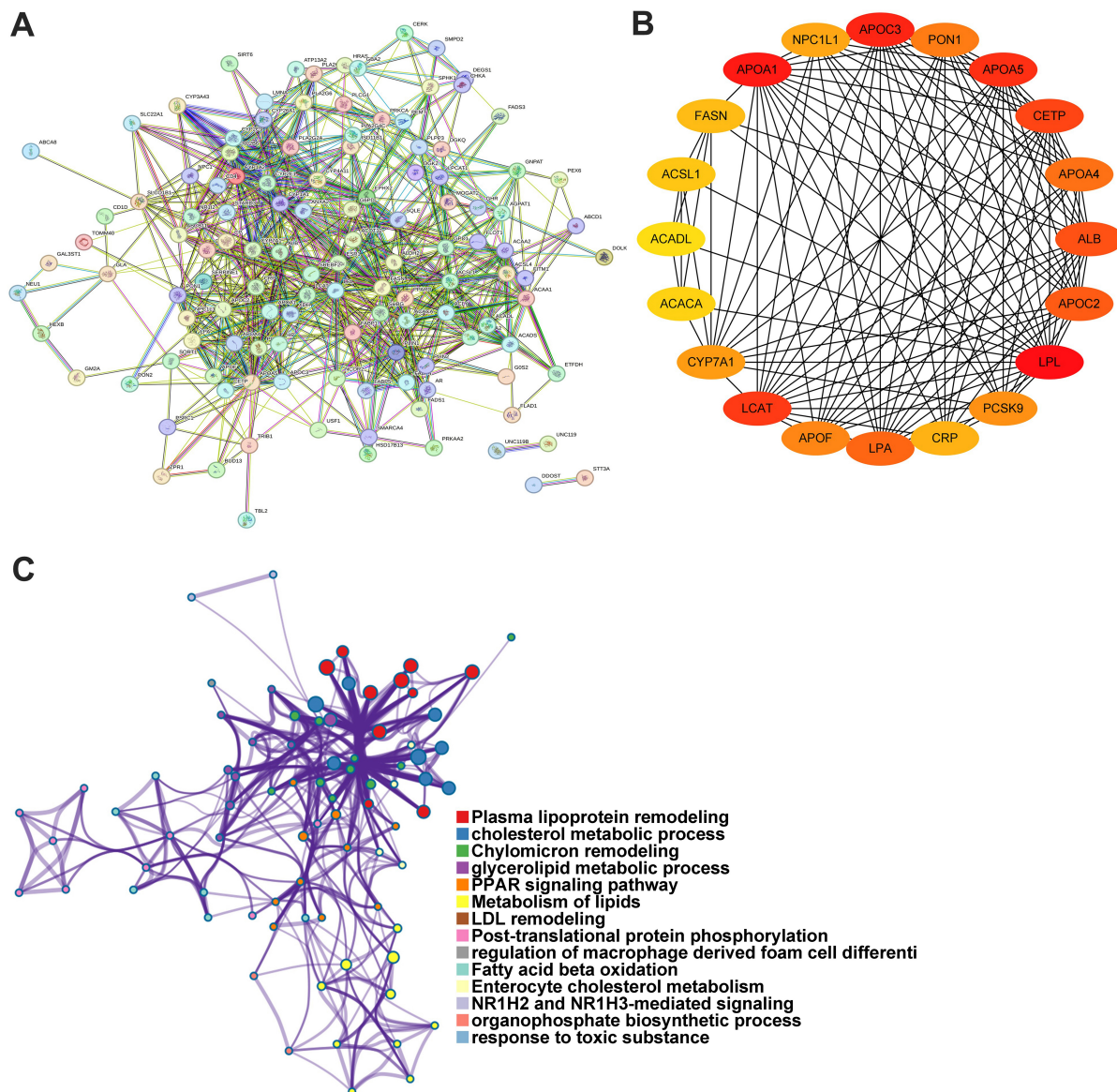


Figure 3. Assessment of the PPI network and recognition of hub Genes. (A) PPI network maps about the 131 DLRGs; (B) STRING analysis network diagram of the top 20 DLRGs obtained using the CytoHubba model in the Cytoscape software; (C) The pathway enrichment analysis of core DLRGs using Metascape. PPI: Protein-protein interaction; DLRGs: differentially expressed lipid-related genes.

Analyzing immune cell infiltration with the application of CIBERSORTx

Lipid metabolism reprogramming plays an important role in immune escaping; however, the precise mechanisms remain clear. In the current study, CIBERSORTx was employed to identify key genes and potential mechanisms through which lipid metabolism mediates immune escape in liver cancer. The association between the expression of the 5 core DLRGs and differentially infiltrated immune cells is shown in [Figure 6A](#). It was found that *LCAT*, *APOC3*, *LPA*, and *PON1* demonstrated a favorable correlation with B cells, CD8+/CD4+ T cells, DCs, Neutrophils, and Tregs, but were negatively associated with macrophages, T

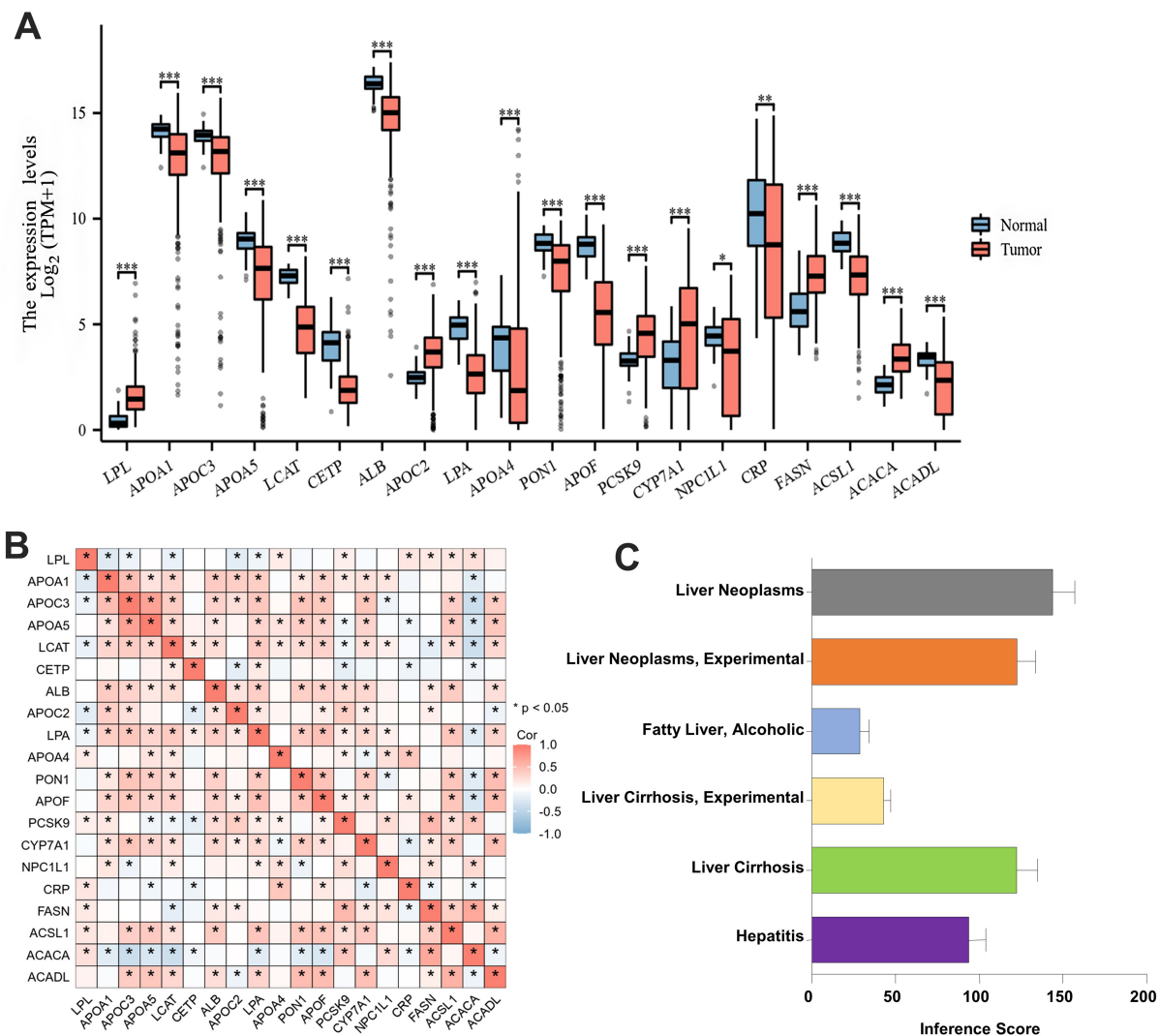


Figure 4. Expression and correlation of the core DLRGs with liver diseases. (A) Expression levels of the 20 core DLRGs according to the TCGA-LIHC database; (B) Heatmap showing the correlations among the 20 core DLRGs; (C) Correlations between the 20 core DLRGs and liver diseases based on data from the CTD. DLRGs: Differentially expressed Lipid-related genes; TCGA: The Cancer Genome Atlas; LIHC: Liver Hepatocellular Carcinoma; CTD: Comparative Toxicomics Database. * $P < 0.05$; *** $P < 0.001$.

helper cells, and Th2 cells. Most interestingly, ACACA almost demonstrated an exactly opposite trend to the other 4 core DLRGs. Using the ssGSEA, the abundances of six infiltrating immune cell types (B cells, cytotoxic cells, CD8+ T cells, neutrophils, macrophages, and dendritic cells), which are related to the 5 core DLRGs (ACACA, LCAT, APOC3, LPA, and PON1), were calculated according to the ggplot2 [3.3.6], stats [4.2.1], and car [3.1-0] R packages. Consistent with what was observed in Figure 6A, ACACA [Figure 6B] was negatively correlated with the abundance of B cells, cytotoxic cells, CD8+ T cells, neutrophils, and dendritic cells. However, LCAT [Figure 6C], APOC3 [Figure 6D], LPA [Figure 6E], and PON1 [Figure 6F] were almost positively correlated with the abundance of the 6 immune infiltrations. Together with the potential prognostic efficacy and the infiltration of immune cells with core DLRGs in HCC, we believe that ACACA might have a more important impact on the progression of HCC cells via mediating lipid metabolism.

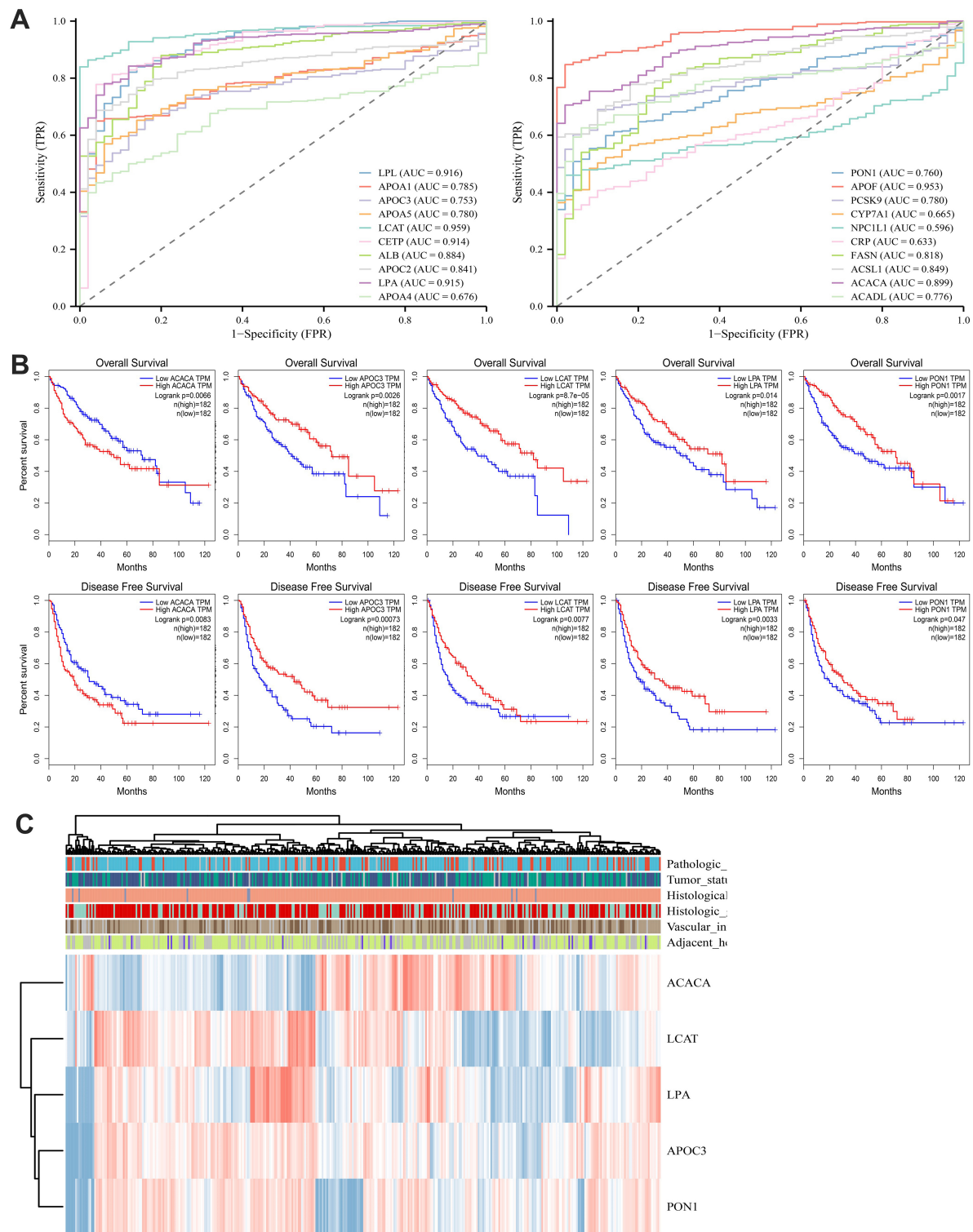


Figure 5. The potential value of core DLRGs for the diagnosis and prognosis of HCC patients. (A) ROC curves of 20 candidate core DLRGs in diagnosing HCC; (B) OS and DFS of the 20 core DLRGs were assessed using Kaplan-Meier analysis according to the TCGA clinical data, and core DLRGs with significant differences in both OS and DFS are shown; (C) Heatmap showing the association between clinical pathological features and the expression levels of ACACA, LCAT, APOC3, LPA, and PON1. DLRGs: Differentially expressed lipid-related genes; HCC: hepatocellular carcinoma; ROC: receiver operating characteristic; OS: overall survival; DFS: disease-free survival; TCGA: The Cancer Genome Atlas.

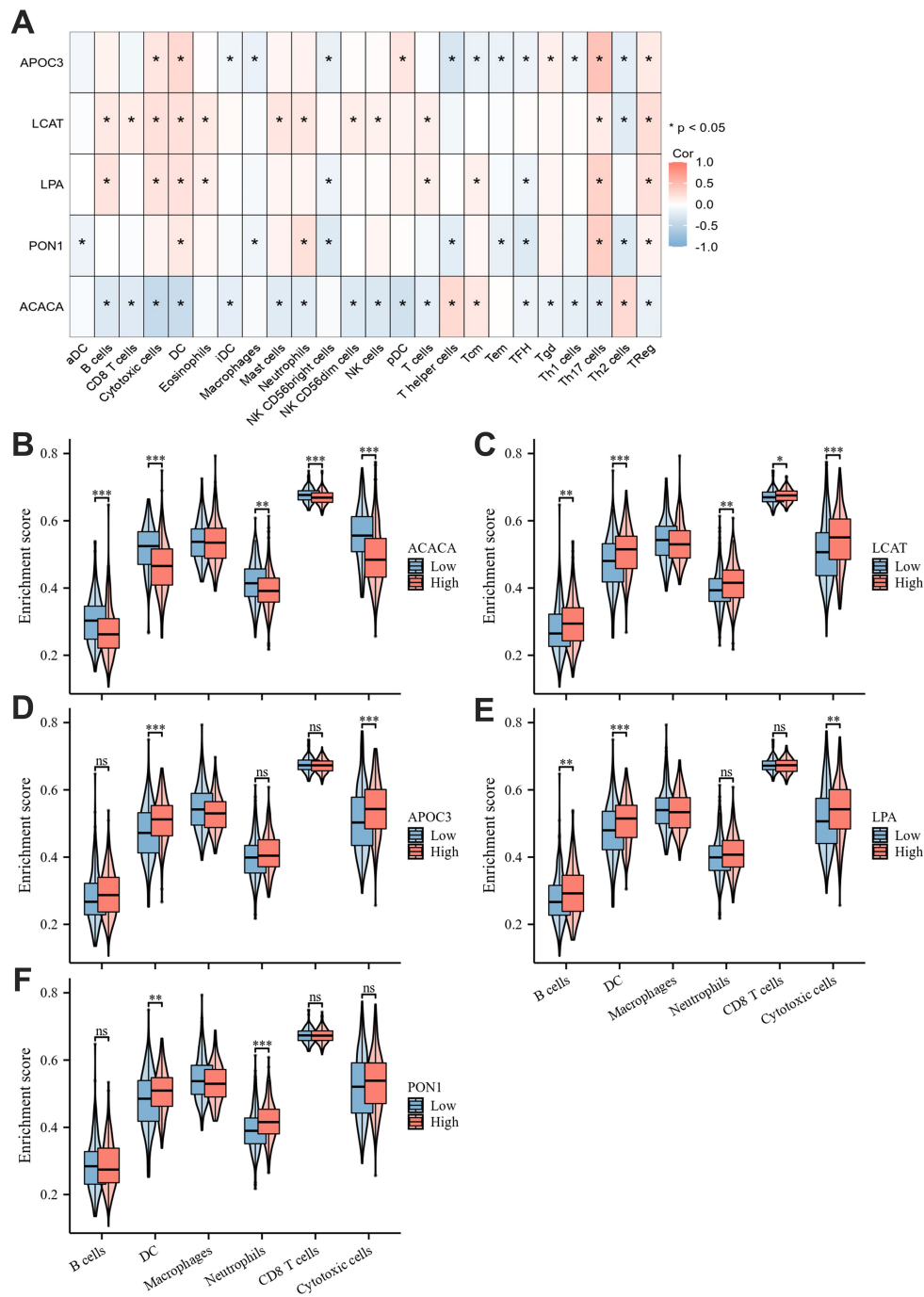


Figure 6. Differentially infiltrated immune cells and core DLRGs. (A) Heatmap illustrating the connection of core DLRGs with infiltrating immune cells. Associations between the expression levels of ACACA (B), LCAT (C), APOC3 (D), LPA (E), and PON1 (F) and the infiltration levels of 6 types of immune cells, as analyzed using the ssGSEA tool. DLRGs: Differentially expressed lipid-related genes; ssGSEA: single-sample gene set enrichment analysis. * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$.

Potential therapeutic agents for lipid metabolism in HCC

Based on the 5 core DLRGs, we identify potential therapeutic agents using the Drug Gene Interactions database. A total of 60 candidate drugs were found to target the 5 core DLRGs, including 11 inhibitors and 35 agonists [Figure 7A]. Regarding the direction of action, 34 agents exhibit activating effects, while 11 show

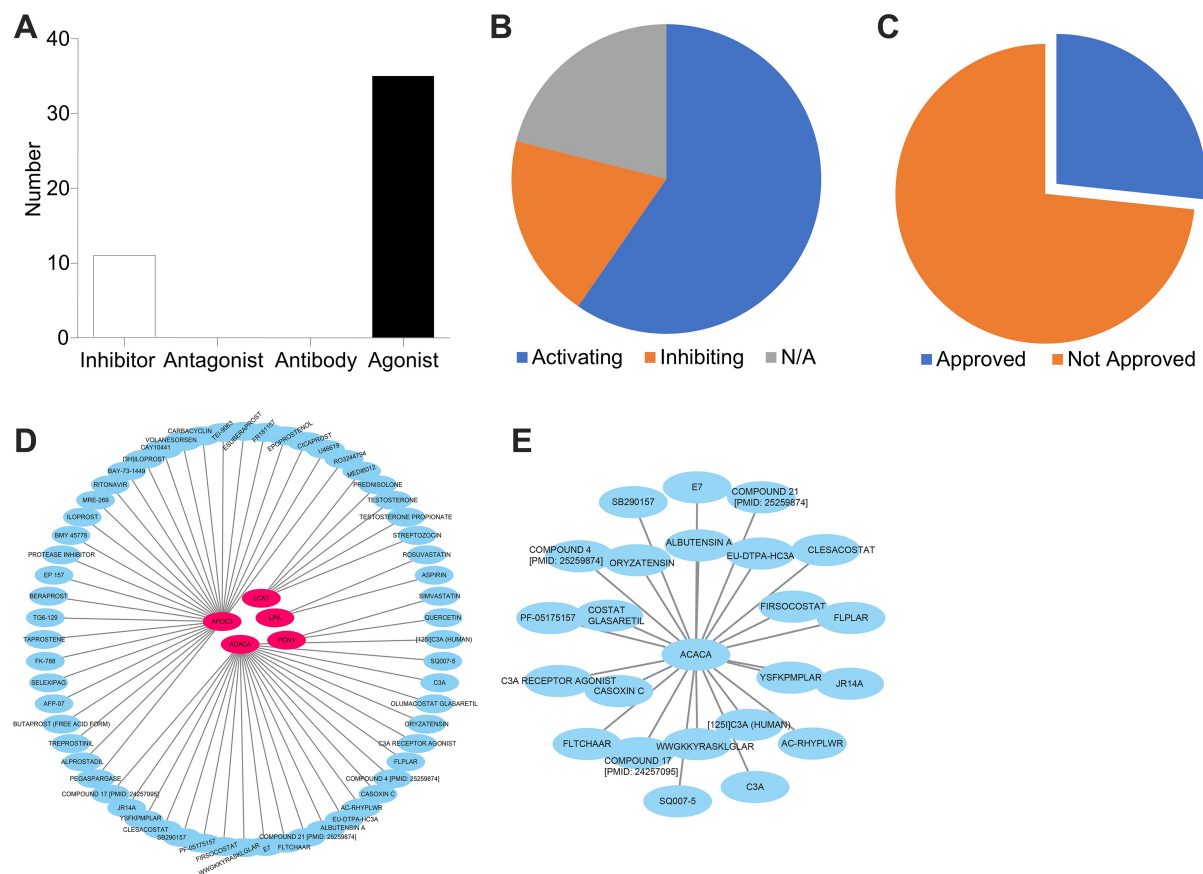


Figure 7. Drug-gene interaction network constructed using Cytoscape. Types of interaction (A), directionality of interactions (B), and (C) FDA-approved drugs targeting the core DLRGs; (D) Network showing the relationships between the 5 core DLRGs (red) and potential drugs (blue); (E) Interactions between the core gene ACACA and potential drugs, based on data from the Drug-Gene Interaction database. DLRGs: Differentially expressed lipid-related genes; FDA: Food and Drug Administration.

inhibitory effects [Figure 7B]. Among the 60 potential drugs, 14 have been approved for clinical use [Figure 7C]. The interaction network between the 5 core DLRGs and the potential drugs was constructed using Cytoscape based on interaction scores, as shown in Figure 7D. Moreover, we found that ACACA and APOC3 are relatively abundant [Supplementary Table 4], and ACACA displayed the most significant interaction with potential drugs [Figure 7E], which is expected to become potential targets for antitumor therapy by targeting lipid metabolism. In addition, we evaluated the potential druggability of the 5 core DLRGs and found that all of them (100%, 5/5) have the potential to be drug targets [Supplementary Table 5]. Notably, ACACA and APOC3 fall into the categories of druggable genome and enzyme, while LCAT and PON1 are classified as transporters, druggable genome, and enzymes. Given that fatty acid synthesis serves as the major source of lipids in liver cancer and that ACACA functions as the key regulatory enzyme in this process, we propose that ACACA plays a vital role in lipid metabolism-mediated tumor progression and represents a promising therapeutic target for the management of HCC.

ACACA is overexpressed and associated with poor OS in HCC

To further investigate the precise role of ACACA, we analyzed data from the TCGA-LIHC database and found that ACACA was highly expressed in a wide range of tumor tissues [Figure 8A]. Additionally, patients with tumors [Figure 8B], advanced pathologic stages T3&T4 [Figure 8C], or stage III&IV [Figure 8D], and higher histologic grades G3&G4 [Figure 8E] demonstrated significantly elevated levels of

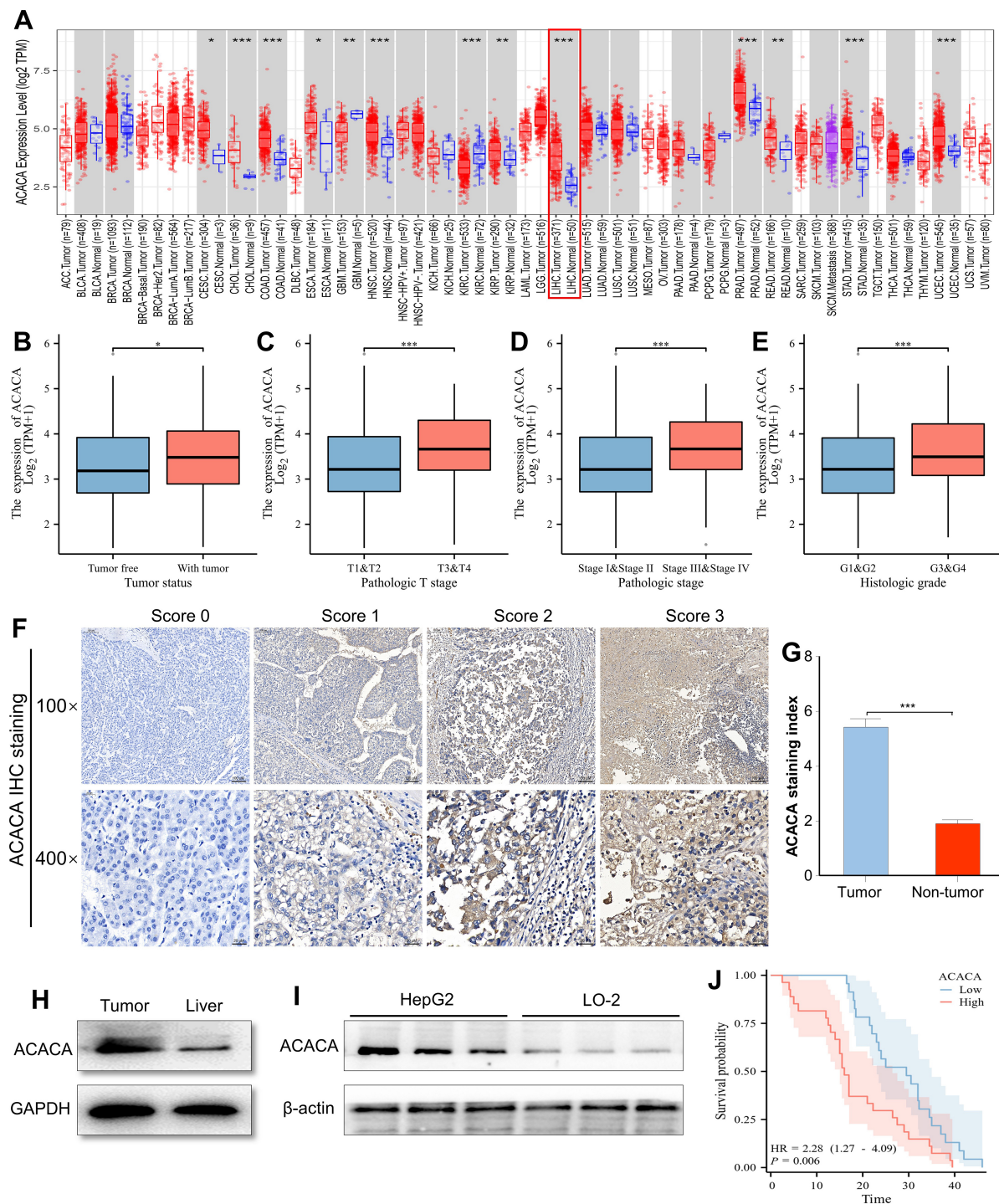


Figure 8. Overexpression of ACACA is correlated with poor overall survival and adverse pathological characteristics in HCC patients. (A) ACACA expression levels in 33 kinds of tumor tissues and their matched non-tumor tissues, analyzed using the TCGA database. (B) ACACA mRNA profiles in HCC tissues compared to adjacent non-tumor tissues, based on TCGA data. ACACA expression across different pathologic T stages (C and D) and histological grade groups (E), according to TCGA. (F) Representative IHC staining images of ACACA in HCC and matched non-tumor tissues, with (G) quantification of IHC staining scores (scale bars: 100 μ m for upper panels, 20 μ m for lower panels). Western blotting analysis of ACACA expression in freshly resected HCC tissues (H) and HCC cell lines (I). (J) Kaplan-Meier analysis comparing OS between patients with high ACACA expression ($n = 27$) and low ACACA expression ($n = 23$). HCC: Hepatocellular carcinoma; TCGA: The Cancer Genome Atlas; IHC: immunohistochemistry; OS: overall survival. * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$.

ACACA compared to the corresponding control groups. Immunohistochemical staining was performed on 85 cases of HCC tissues and their paired adjacent liver tissues [Figure 8F]. The IHC analysis revealed that ACACA expression was significantly higher in tumor tissues compared to normal liver tissues [Figure 8G]. The expression of ACACA with the clinicopathological features (such as age, sex, history of cirrhosis, clinical stage, AFP level or tumor size) of HCC patients was also measured; however, no significant difference was observed [Supplementary Table 6]. We also measured the expression of ACACA in 5 cases of freshly resected HCC tissues and matched normal tissues by Western blotting, and found that the expression of ACACA was significantly upregulated in HCC tissues compared with non-tumor tissues [Figure 8H]. Moreover, the expression of ACACA was also increased in HepG2 cells compared to human hepatocyte LO-2 cells [Figure 8I]. Most interestingly, we explored the relationship between ACACA expression and OS in 50 cases of HCC patients. As revealed by the Kaplan-Meier survival analysis, HCC patients with evaluated levels of ACACA had poorer prognosis [Figure 8]. These findings hint that the potential function of ACACA is closely linked to the development and progression of HCC.

Activation of ER stress positively correlated with upregulation of ACACA in HCC

Studies have revealed that ER stress is universally activated in various solid tumors, such as HCC^[18], and the activation of ER stress plays a crucial role in the progression of NASH-associated HCC by setting off chronic liver injury and inflammation^[19]. Thus, we wonder whether ER stress promotes the development of HCC via modulating ACACA-mediated fatty acid synthesis. We first measured several ER stress-related markers (PERK, IRE1 α , ATF6, and GRP78) by Western blotting analysis, and found that ER stress was activated in HCC tissues in comparison with matched non-tumor tissues [Figure 9A]. Immunohistochemical analysis also revealed a substantial upregulation of GRP78 in HCC tissues compared to matched non-tumor tissues [Figure 9B]. Moreover, we stimulated HepG2 cells with different concentrations of free fatty acid (FFA) and found FFA could dose-dependently activate ER stress [Figure 9C]. Interestingly, we found tunicamycin (TM, a known ER stress inducer) could dose-dependently increase the expression of ACACA [Figure 9D]. TCGA database also showed that ACACA was positively correlated with several ER stress marker proteins, such as *ERN1*, *HSPA5*, *EIF2AK3*, *ATF6*, *ATF4*, *DDIT3*, and *XBP1* [Figure 9E]. Taken together, our results suggest that ER stress might modulate lipid metabolism in HCC via ACACA, and ACACA might turn out to be a promising target for treating liver cancer.

ACACA promotes HCC cell proliferation

To explore the significance of ACACA in the evolution of HCC and find potential therapeutic agents, we first stimulated HepG2 cells with TM and PA, respectively, and then Western blotting analysis showed that both TM [Figure 10A] and PA [Figure 10B] significantly increased the expression of ACACA, while treating with fenofibrate (100 μ M) for 24 h could significantly reverse the effects of TM and PA. In accordance with Western blotting analysis, qRT-PCR assays showed that fenofibrate could not only decrease PA-induced upregulation of ACACA [Figure 10C], but also decrease the expression of *FASN* [Figure 10D], *PPAR- γ* [Figure 10E], and *C/EBP- β* [Figure 10F]. Moreover, we found that 4-phenylbutyric acid (4-PBA), an inhibitor of HDAC and ER stress, has the same effect as fenofibrate on TM-induced upregulation of ACACA [Figure 10G]. These results indicate that activation of ER stress could enhance fatty acid synthesis via upregulating ACACA in HCC, and inhibition of ER stress represents a promising way of preventing the development of liver cancers. Thus, we went on to explore the function of fenofibrate on the proliferation of HCC cells and found that PA could significantly increase HepG2 cell viability, while fenofibrate significantly decreased HepG2 cell viability under the stimulation of PA [Figure 10H]. Overall, our findings indicate that ER stress might promote HCC cell proliferation via enhancing ACACA-mediated fatty acid synthesis.

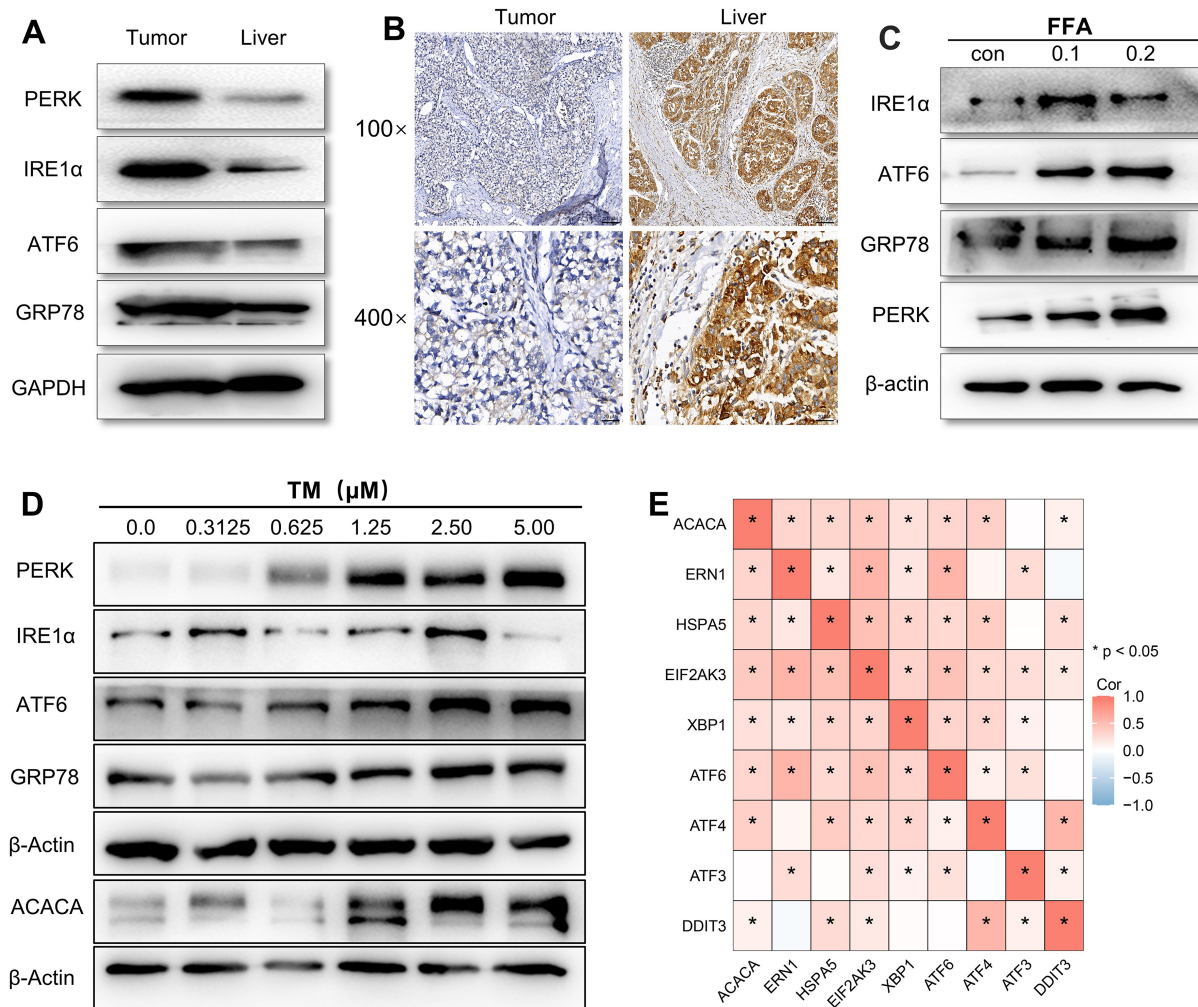


Figure 9. Activation of ER stress shows a positive association with the upregulation of ACACA in HCC. (A) Western blotting analysis of the biomarkers of ER stress in HCC tissues and paired non-tumor tissues; (B) Typical immunohistochemistry images of GRP78 protein in HCC tissues and paired liver tissues (scale bars: 100 μ m for upper panels, 20 μ m for lower panels); (C) Western blotting examination of ER stress markers after co-incubation with different concentrations of FFA for 24 h; (D) Western blotting analysis of the expression of ER stress markers and ACACA in HepG2 cells after co-incubation with different concentrations of TM for 24 h; (E) Heatmap of the correlation of the ER stress marker genes and ACACA according to TCGA-LIHC databases. ER: Endoplasmic reticulum; HCC: Hepatocellular carcinoma; FFA: free fatty acids; TM: tunicamycin; TCGA: The Cancer Genome Atlas; LIHC: Liver Hepatocellular Carcinoma. * $P < 0.05$.

DISCUSSION

The pathogenesis and progression of HCC is a multifactorial systematic and multistep process. The liver, an important organ for lipid metabolism, undergoes abnormal metabolic changes throughout the liver lesions^[20]. It has been discovered that normal cells predominantly obtain lipids through exogenous uptake, whereas tumor cells mainly rely on DNL to maintain lipid homeostasis, and the increase in DNL is a common characteristic of human tumors^[21]. Previous studies on HCC have revealed several alterations, including enhanced *de novo* fatty acids biosynthesis, decreased oxidative states, elevated secretion of insulin and insulin-like growth factor, and disrupted phosphatidylcholine metabolism^[22]. These metabolic pathways furnish intermediate energy substances that facilitate the growth, proliferation, and metastasis of HCC cells. Furthermore, pharmacological or genetic modulation of the FA metabolizing enzymes, such as FASN, short-chain acyl-CoA dehydrogenase, and long-chain acyl-CoA dehydrogenase, significantly influences

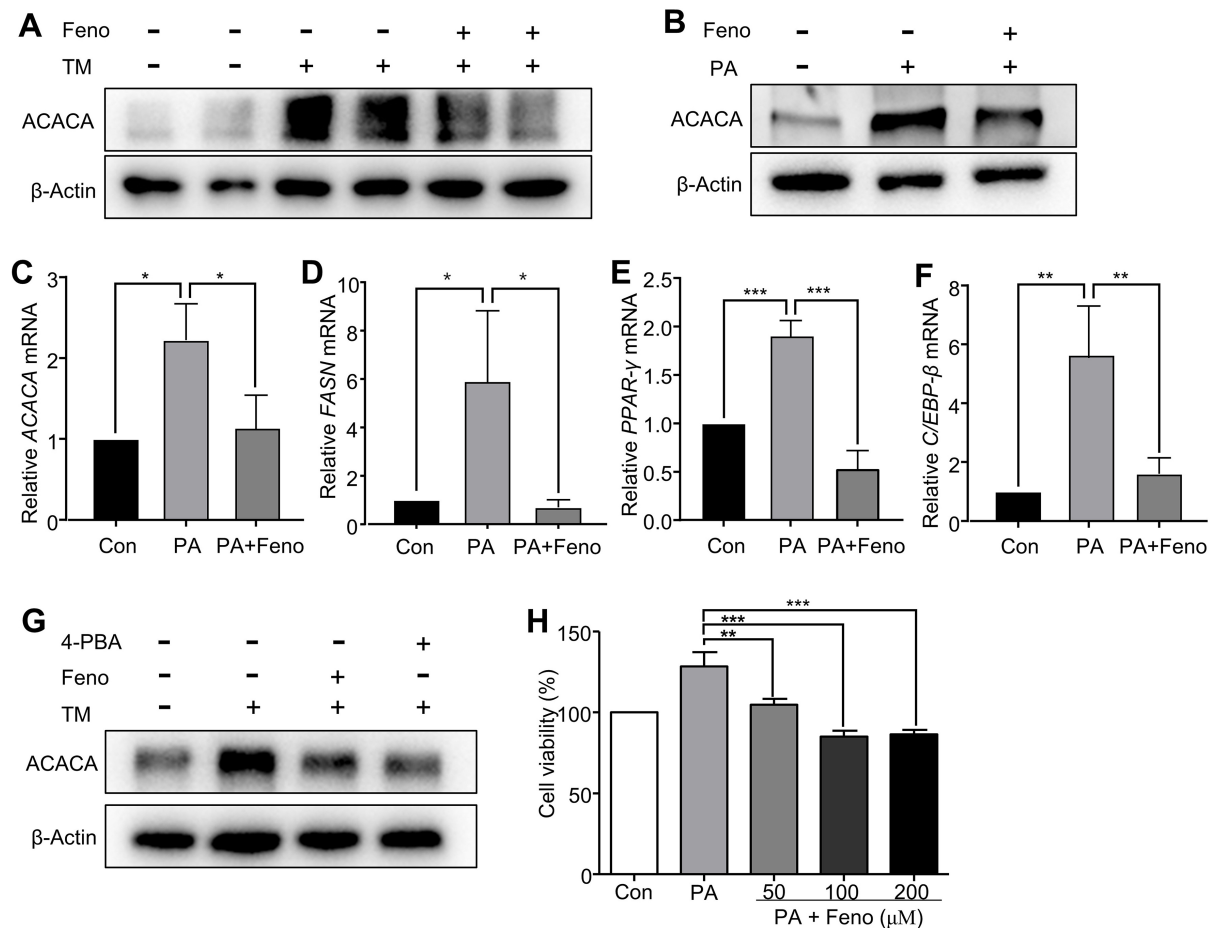


Figure 10. ACACA promotes proliferation in HCC. Utilization of Western blotting to analyze ACACA expression in HepG2 cells treated with TM (A) or a combination of PA and fenofibrate (B). qRT-PCR assays measured the expression of ACACA (C), FASN (D), PPAR-γ (E), and C/EBP-β (F) following treatment with PA and fenofibrate. (G) Western blotting analysis of the effects of fenofibrate or 4-PBA on ACACA expression levels in TM-treated HepG2 cells. (H) Cell viability was assessed using the CCK-8 assay. HCC: Hepatocellular carcinoma; TM: tunicamycin; PA: palmitic acid; qRT-PCR: quantitative real-time polymerase chain reaction; 4-PBA: 4-phenylbutyric acid; CCK-8: Cell counting kit-8. * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$.

tumor growth, invasion, and metastasis, suggesting that altered lipid metabolism plays a role in HCC development^[23]. Additionally, it has been noted that lipid metabolism is associated with chemotherapy insensitivity^[24], and these studies highlight the significance of targeting fatty acid synthesis as a potential strategy for antitumor therapy. Lipogenesis is pronounced in those suffering from NASH, and its elevation is correlated with a greater risk and dismal prognosis of NASH-associated HCC^[25]. In this study, 131 DLRGs were identified using data from the TCGA-LIHC and Genecards databases. Twenty genes closely related to lipid metabolism in HCC were screened by GO, KEGG, and PPI analyses, of which *ACACA*, *APOC3*, *LCAT*, *LPA*, and *PON1* had a tight association with the prognosis of HCC, as determined using the survival package in R software. ROC curves revealed that the AUC corresponding to the five core DLRGs was above 0.75, indicating that the genes screened in the study had a favorable predictive ability to prognosticate liver cancer outcomes. Nevertheless, the abnormalities of lipid metabolism in HCC, particularly the genes associated with lipid metabolism, remain incompletely understood in terms of their functions in the development, diagnosis and management of HCC.

Enhanced lipogenesis is characterized by heightened activity and expression of multiple lipogenic enzymes, such as adenosine triphosphate (ATP) citrate lyase (ACLY), ACACA, and FASN. These enzymes are highly expressed in a wide range of cancers, including HCC. Dysregulation of genes involved in lipid metabolism has been linked to tumorigenesis and the progression of HCC. For example, ADH1A triggers the malignant transformation of hepatocytes and is associated with poor survival^[26], while the extracellular form of PEDF impedes tumor angiogenesis by disrupting lipid metabolism^[27]. acetyl-CoA carboxylase (ACC), a rate-limiting enzyme in fatty acid synthesis, plays a key role in enhancing hepatic DNL during HCC progression. ACC facilitates the transformation of acetyl-CoA to malonyl-CoA in the cytoplasm; however, phosphorylated ACC reduces hepatic malonyl-CoA levels and attenuates the progression of NAFLD and liver fibrosis^[28]. ND-654, a specific ACC inhibitor, has been shown to suppress hepatic DNL and inhibit the development of HCC^[29]. FASN is also involved in HCC progression, and pharmacological inhibition of FASN significantly impairs tumor cell proliferation^[30]. In this study, we found that several genes closely related to fatty acid metabolism, including ACACA, FASN, ACLY, and ACSL4, were significantly dysregulated. Moreover, genes involved in cholesterol and lipoprotein metabolism, such as LCAT and APOC, were also identified. These insights into the molecular mechanisms and metabolic alterations underlying HCC pathogenesis highlight the potential of metabolism-related enzymes and pathways as biomarkers for the diagnosis and treatment of HCC.

Tumor cells are frequently exposed to microenvironmental stimuli that result in ER stress. It has been noted that tumor cells developed the capacity to reconfigure ER stress-associated pathways to sustain their malignant behaviors^[31]. The initiation of the ER stress signaling pathway plays a critical role in the progression of NASH-associated HCC by provoking persistent liver damage and inciting inflammation^[19,32]. In this study, we found proteins associated with ER stress, including GRP78, PERK, and ATF6, were significantly increased in HCC tissues in comparison with the matched non-tumor tissues, and were associated with an unfavorable prognosis for individuals with HCC, which was consistent with our previous report. Cancer cells show a greater capacity to commandeer the ER stress pathway to facilitate malignant behavior in response to severe stimuli. Upon ER stress, IRE1 α phosphorylates SEC63 to enhance ACLY stability, which increases acetyl-CoA and lipid biosynthesis, thereby enhancing ER capacity for HCC survival^[33]. FASN and ACACA serve as the crucial enzyme involved in lipid metabolism. Upon ER stress, the transcriptional activity of SREBP-1c is enhanced, upregulating the expression levels of FASN and ACACA and thus increasing the synthesis of fatty acid and triglyceride^[34]. In the current study, ACACA showed considerably higher levels in HCC tissues when contrasted with matched non-tumor tissues, and findings from TCGA-LIHC analysis revealed that the expression of ACACA was correlated with tumor status, pathological stages, and pathological grades. Notably, ACACA expression was higher in HCC cells than in normal hepatocytes, and it showed a positive correlation with the expression levels of most ER stress markers. Additionally, we induced a fatty liver model in HepG2 cells using FFA and found a marked increase in both mRNA and protein related to lipid synthesis, as well as in ER stress markers, consistent with previous findings^[35]. Moreover, our earlier work demonstrated that ER stress upregulates the expression of pyruvate kinase isoform M2 (PKM2) via the miR-188-5p/hnRNPA2B1 axis in HCC cells^[36], suggesting the involvement of a metabolism-regulating pathway that is independent of the UPR. Collectively, these results suggest that ER stress may regulate lipid metabolism-related enzymes in HCC through both UPR-dependent and -independent mechanisms.

Acetyl-CoA serves as a central metabolic intermediate and substrate for DNL, supporting lipid biosynthesis, which is upregulated in response to ER stress^[37]. ACACA functions as the pivotal rate-limiting enzyme responsible for catalyzing the ATP-dependent carboxylation of acetyl-CoA. Furthermore, the heterogeneity of FA synthesis among different HCC cells is regulated by ACACA expression. This heterogeneity is evident

as HCC cells overexpressing ACACA exhibit enhanced glucose utilization for DNL, leading to increased lipid accumulation within the cells. Moreover, ACACA overexpression is related to poor prognosis in various human malignancies, such as lung cancer and HCC^[38,39]. Our observations confirmed that ACACA is highly expressed in HCC tissues, and elevated ACACA levels predicted poor OS in a cohort of 50 HCC patients. We also found that TM increased both ER stress biomarkers and ACACA in a dose-dependent manner. Therefore, understanding the regulatory mechanisms of ACACA following ER stress activation in HCC cells is of significant interest. Herein, we demonstrated that 4-PBA, an ER stress inhibitor, significantly reduced ACACA expression in HepG2 cells, but the precise underlying mechanism needs further exploration. Most notably, fenofibrate markedly suppressed TM- and FFA-induced upregulation of ACACA, as well as the expression of FASN, PPAR- γ , and C/EBP- β . These findings underscore the crucial role of ACACA in modulating FA metabolism in HCC under ER stress conditions.

Given the role of ACACA in DNL, targeting ACACA as a therapeutic strategy is particularly important for various cancers, including HCC^[29,40]. Although ACC inhibition has been investigated preclinically in several cancer types, there is limited evidence specifically within HCC models^[41]. Notably, ND-654 significantly reduced HCC incidence by up to 41%, a reduction comparable to that achieved by single-agent sorafenib (a drop of 57%). When ND-654 was combined with sorafenib, the incidence of HCC was further reduced by 81%^[29]. Unfortunately, these drugs exhibit unfavorable toxicity profiles, limiting their clinical use as effective treatment options. Using the DGIdb, we identified 60 potential agents targeting core DLRGs (ACACA, APOC3, LCAT, LPA, and PON1), among which 23 are expected to target ACACA. However, none of these agents have received FDA approval. Importantly, we found that fenofibrate inhibits the expression of lipid metabolism-related enzymes ACACA at both transcriptional (mRNA) and translational (protein) levels and impedes PA-induced proliferation of HepG2 cells. Thus, exploring fenofibrate's potential as a treatment for HCC, especially in patients with concurrent hyperlipidemia, would be highly valuable in future studies.

There are several limitations in the current study. First, our clinical validation involved only 85 cases of liver cancer patients, which is relatively small and may not fully reflect the molecular heterogeneity of liver cancer. This limits the generalizability of the observed relationship between gene expression and ER stress across the broader HCC population. Second, the biological functions of ACACA, such as its role in apoptosis during ER stress and lipid metabolism abnormalities, have not been verified through gene knockout/overexpression experiments, making it difficult to establish a causal link. Finally, while the study confirmed a correlation between ACACA expression and ER stress markers (e.g., GRP78), it did not investigate whether ER stress directly regulates ACACA expression via transcription factors or indirectly affects its activity through lipid metabolic reprogramming. The lack of in-depth studies on post-transcriptional gene modification, PPI, and specific upstream and downstream targets and regulatory pathways involving ACACA has constrained a comprehensive understanding of the mechanisms connecting ER stress and abnormal lipid metabolism in the development of liver cancer.

In summary, we identified five core DLRGs (including ACACA, APOC3, LCAT, LPA, and PON1) as potential diagnostic and prognostic markers for HCC. Among these, ACACA was significantly upregulated in HCC tissues compared to matched non-tumor tissues, and was negatively correlated with OS and immune cell infiltration. FFA was found to activate ER stress, and a positive correlation existed between ER stress activation and ACACA levels in HCC. Moreover, Drug-Gene interaction analysis suggested that ACACA could serve as a potential therapeutic target for HCC. Most importantly, we found that fenofibrate inhibited TM-induced upregulation of ACACA and decreased cell viability in HCC cells. These findings might contribute to a deeper understanding of markers for the diagnosis and treatment of HCC.

DECLARATIONS

Acknowledgments

The authors would like to thank the Center for Scientific Research of Anhui Medical University for the valuable help in the experiment.

Authors' contributions

Data curation, formal analysis, investigation, methodology, validation, writing - original draft, writing - review and editing: Liu X

Investigation, methodology, project administration: Li S

Methodology, formal analysis, resources, data curation: Rao P, Yu W, Tang Y

Resources, data curation: Wang A

Conceptualization, formal analysis, resources, data curation: Chen M, Sun G

Funding acquisition, conceptualization, resources, supervision, writing - review and editing: Liu J

Availability of data and materials

The original contributions presented in the study are included in the article/[Supplementary Materials](#). Further inquiries can be directed to the corresponding author.

Financial support and sponsorship

This study was supported by the National Nature and Science Foundation of China (grant no. 82072687) and the Anhui Provincial Natural Science Foundation (grant no. 2008085MH257).

Conflicts of interest

All authors declared that there are no conflicts of interest.

Ethical approval and consent to participate

This study protocol conforms to the ethical guidelines outlined in the Declaration of Helsinki (1975) and was approved by the Ethics Committee of Anhui Medical University (20180406). All clinical specimens were collected from patients who provided written informed consent.

Consent for publication

Not applicable.

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