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AIMS AND SCOPE

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ORIGINAL ARTICLE

Basic Study

Carnitine palmitoyltransferase-II inactivity promotes malignant progression of metabolic dysfunction-associated fatty liver disease *via* liver cancer stem cell activation

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Abstract

BACKGROUND

Metabolic dysfunction-associated fatty liver disease (MAFLD) is one of the main chronic liver diseases. However, the roles of mitochondrial carnitine palmitoyl transferase-II (CPT-II) downregulation and liver cancer stem cell (LCSC) activation remain to be identified.

AIM

To investigate the dynamic alterations in CPT-II inactivity and LCSC activation during the malignant progression of MAFLD.

METHODS

Dynamic models of mouse MAFLD were generated *via* the consumption of a high-fat diet or the addition of 2-fluorenylacetamide for hepatocarcinogenesis. The mice were divided into groups on the basis of hematoxylin and eosin staining. Biochemistries, CPT-II, intrahepatic T cells, and LCSCs were determined and confirmed in clinical samples. The mitochondrial membrane potential (MMP) was analyzed. Differentially expressed genes were screened *via* RNA sequencing and enriched in KEGG pathways or GO functions.

RESULTS
Dynamic models of MAFLD malignant transformation were successfully generated on the basis of pathological examination. Hepatic lipid accumulation was associated with the loss of mitochondrial CPT-II activity and alterations in the MMP, with decreases in liver CD3+ or CD4+ T cells and increased AFP levels. In the lipid accumulation microenvironment, mitochondrial CPT-II was inactivated, followed by aberrant activation of CD44+ or CD24+ LCSCs, as validated in MAFLD or hepatocellular carcinoma patient samples. In terms of mechanism, the biological process category focused mainly on the metabolic regulation of cells in response to external stimuli. The enriched molecular functions included protein binding, cell apoptosis, and cell proliferation.

CONCLUSION
CPT-II inactivity promotes the malignant progression of MAFLD *via* the loss of innate immune function and abnormal LCSC activation.

Key Words: Metabolic dysfunction-associated fatty liver disease; Carnitine palmitoyl transferase-II; Mitochondria; T lymphocytes; Liver cancer stem cells

Core Tip: Metabolic dysfunction-associated fatty liver disease (MAFLD) is one of the main chronic liver diseases. The present study investigated the dynamic alterations in mitochondrial carnitine palmitoyl transferase-II (CPT-II) in T cells and liver cancer stem cell (LCSC) activation in MAFLD during hepatocyte malignant progression under lipid accumulation. There was a loss of mitochondrial CPT-II activity and a decrease in the mitochondrial membrane potential with decreasing numbers of CD3+ or CD4+ T cells. Mechanistically, CPT-II inactivity *via* the loss of innate immune function with abnormal activation of LCSCs promotes the malignant progression of MAFLD. These results suggest that CPT-II prevents lipid accumulation and LCSC activation, as well as improves IL-CD3/CD4 T cell function for hepatocellular carcinoma immunotherapy and delays MAFLD malignant progression.

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INTRODUCTION

Metabolic dysfunction-associated fatty liver disease (MAFLD) is a major health burden, and its incidence is increasing worldwide[1,2]. An imbalance in lipid accumulation in MAFLD may cause inflammatory reactions that lead to liver fibrosis, metabolic dysfunction-associated steatohepatitis (MASH), or hepatocellular carcinoma (HCC)[3,4]. Mitochondrial dysfunction has been observed in MASH patients and animal models[5], and it is recognized as an important factor, including damage to mitochondrial DNA (mtDNA) or direct blockade of fatty acid β-oxidation (FAO)[6]. Few studies have shown that carnitine palmitoyl transferase-II (EC 2.3.1.21, CPT-II) on the inner mitochondrial membrane (IMM) may be an early monitoring marker of MAFLD or related liver cirrhosis (LC) and HCC[7-9]. As an important member of the mitochondrial FAO respiratory chain, the CPT-II gene (*CPT2*) is related to energy generation disorders, virus replication, and inflammation[10], and its variants inhibit CPT-II activity to affect the carnitine transport of long-chain fatty acids and ATP production[11,12]. CPT-II is downregulated in MASH/HCC *via* metabolic reprogramming and has been confirmed to adapt to a fat-rich environment. However, lipotoxicity may affect FAO, leading to liver steatosis and directly contributing to HCC. Moreover, the malignant transformation of hepatocytes may prevent lipotoxicity, and the regulation of lipocarnitine-activated signal transduction and activator of transcription 3 or interleukin-6 (IL-6) enhances the self-renewal ability of HCC cells and gradually promotes HCC progression[13,14].

MAFLD encompasses a spectrum of hepatocyte lesions, including metabolic dysfunction-associated fatty liver (MAFL), MASH, and LC. Differentially abundant metabolites[15] or the adaptive immune response[16] are important parts of MAFLD. In MAFLD models, excessive hepatic lipids have a significant effect on intrahepatic CD4+ T lymphocytes (IL-CD4), which produce more reactive oxygen species (ROS) compared to IL-CD8, and blocking ROS *in vivo* may partially prevent the loss of IL-CD4 and delay MAFLD progression[17-19]. Activated T cells are involved in lipid metabolic reprogramming and upregulate lipid synthesis and cholesterol uptake. Innate immune mechanisms represent a key element in supporting hepatic inflammation in MASH patients[17]. However, the specific regulatory mechanism involved in the T lymphocyte immune response in MAFLD and the exact relationship between CPT-II and IL-CD4 have yet to be identified[20,21]. In the present study, the dynamic changes in mitochondrial CPT-II and T cells during MAFLD progression were investigated, with a focus on liver cancer stem cell (LCSC) activation.

MATERIALS AND METHODS

Data acquisition

After approval from the database of Genotypes and Phenotypes access committee, the raw RNA sequencing datasets and information for LIHC patients were extracted from TCGA (https://portal.gdc.cancer.gov) database[22]. CPT-II transcript levels (*CPT2)* were detected in LIHC tissues (n = 269), normal livers (n = 50), self-controlled LIHC tissues (n = 50), and self-controlled non-HCC tissues (n = 50). CPT-II levels were analyzed and visualized *via* the GGPLOT2 package.

Liver tissues and blood

Blood and 12 matched postoperative HCC and non-HCC tissues were collected from HCC patients with MAFLD from May 2020 to April 2023. The present study was approved by the independent Ethics Committee of The Affiliated Hospital of Nantong University, China (NTU-2021-43). Informed consent was obtained from all patients who had not undergone preoperative chemotherapy or radiotherapy in accordance with the Helsinki Declaration. Livers were confirmed by pathological examination with hematoxylin & eosin (H&E) staining.

Model design

The use of dynamic hepatocyte models under lipid accumulation conditions was approved (S20200318-017) by the guidelines of the Animal Care and Use Committee of Nantong University, China. Male C57BL/6 (B6) wild-type littermates (6 weeks old) were fed a high-fat diet (HFD; 43.7% fat, 36.6% carbohydrate, 19.7% protein, 0.2% cholesterol) for MAFLD, a HFD with 0.05% 2-fluorenylacetamide (2-FAA, Sigma, United States) was used to induce HCC formation. The normal control (NC, 18% fat, 58% carbohydrate, 24% protein) diet was fed for 16 weeks[21]. The mice were monitored at different times and sacrificed, and blood was collected every two weeks. After the mice were sacrificed, livers were collected and evaluated by H&E staining. The samples were divided into MAFL, MASH, LC, HCC, and NC groups. Lipids were stained with oil red O.

Transcriptome sequencing

T cells were collected and lysed with TRIzol, and total RNA was extracted and reverse transcribed into cDNA. The sequencing libraries were constructed after PCR amplification. Transcriptome sequencing was performed after passing quality control, and differentially expressed genes (DEGs) were screened by genome matching and subjected to subsequent analysis.

DEGs with functional enrichment

DEG screening data from T cell subgroups at different stages were used for functional analysis. Gene Ontology (GO) was used to annotate cellular components, molecular functions, and biological processes. In addition, GO or KEGG enrichment analysis of the DEGs was performed *via* the clusterProfiler R package. Hub genes were screened *via* the Cytohubba plugin, and the top 10 genes were selected as Hub genes *via* the MCC algorithm.

Cell sorting and flow cytometry

To sort the T cell populations *via* FACS, the livers were dissected and finely minced in 0.25% collagenase (Sigma-Aldrich) in HBSS (Gibco, United States), incubated at 37 °C for 20 minutes, and digested in 10 mL of 0.25% trypsin/EDTA (Gibco, United States) for 10 minutes at 37 °C. Trypsin was then quenched with 10 mL of FACS buffer [5% FCS, 10 mmol/L EDTA, and 1 mmol/L HEPES in phosphate-buffered saline (PBS)], after which the mixture was centrifuged, resuspended in 20 mL of FACS buffer, and strained through a 70 μm cell strainer (BD Biosciences). The filtered samples were centrifuged, and the supernatant was discarded. The cells were then resuspended in a cocktail of the following primary antibodies against surface markers at predetermined levels: CD3-APC (1:100; BioLegend), CD45-APC (1:200; BioLegend), CD8-APC (1:100; BioLegend), CD24-APC (1:250; Thermo Fisher Scientific), and CD44-PECy7 (1: 100; BD Biosciences). The primary antibody cocktail was prepared in buffer with 100 ng/mL DAPI on ice. In brief, the lymphocytes were counted, and the concentration was adjusted. Next, 100 μL of the cell suspension was removed, and the mixture was incubated with antibodies on ice. The centrifuge tube was shaken well while avoiding light for 15 minutes, and the mixture was subsequently washed with cold PBS containing 2% fetal bovine serum. After centrifugation, the supernatant was discarded, and the cells were resuspended in 100 mL of staining buffer. CD3+, CD4+, CD4+/CD8+, and CD44+ or CD24+ T cells were measured with a FACSCanto-Flow cytometer (BD Company, United States).

Western blot analysis

Livers were isolated, flushed with cold PBS, weighed, shredded, homogenized in radioimmunoprecipitation assay (RIPA, Sigma-Aldrich, Shanghai, China) buffer with protease and phosphatase inhibitors, and centrifuged at 4 °C for 4 minutes. The supernatants were then collected, and the protein levels were measured. Standards or samples were added to 2 × loading buffer at 100 °C for 5 minutes and then electrophoresed on polyacrylamide gels. The proteins were transferred to membranes at 90 V on ice for 90 minutes, and the membranes were blocked with 5% skim milk on a shaker at 25 °C for 2 hours. The membranes were washed with Tris-HCl-buffered saline with Tween (TBST, Shanghai, China) and incubated with diluted primary antibodies (1:1000) overnight on a shaker at 4 °C. After the primary antibodies were removed, the membranes were incubated with diluted secondary antibodies (1:2000) in 5% skim milk at 25 °C for 2 hours. The membranes were then washed three times with TBST and developed with ECL. The developer parameters were adjusted as necessary.

RNA isolation and RT-PCR

Total RNA was extracted from livers in RNase-free tubes containing RNAlater (Ambion, TX, United States) with TRIzol reagent (Gibco BRL, United States). Random primers and total RNA (1 μg/μL) were used to generate cDNA with reverse transcriptase (Gibco BRL). PCR amplification of *CPT2* was performed according to previously described methods[5].

Mitochondrial membrane potential

T cells were cultured at 37 °C on glass chamber slides (Fisher Scientific, Leicestershire, United Kingdom), treated with bezafibrate for 24 hours, incubated for 15 minutes with 10 μM 5,5′,6,6′-tetrachloro-1,1′,3,3′-tetraethyl benzimidazolyl carbocyanine iodide fluorescent dye (JC-1, Molecular Probes, Paisley, United Kingdom) in the dark, washed according to the protocol of the manufacturer, and immediately imaged *via* a fluorescence microscope (Keyence, Osaka, Japan) with red (λ: 560 ± 40 nm, λ: 630 ± 60 nm) and green fluorescence (λ: 470 ± 40 nm, λ: 535 ± 50 nm)[5].

Immunohistochemistry

Paraffin-embedded liver sections were dewaxed, rehydrated, subjected to antigen retrieval, incubated with 3% H_2O_2 for 10 minutes, and then incubated with rabbit anti-mouse monoclonal CPT-II antibodies (Ab181114, diluted 1:80; Abcam, United Kingdom) at 4 °C overnight. After being washed, reaction enhancement solution was added to the sections for 20 minutes, followed by incubation with horseradish peroxidase-conjugated goat anti-rabbit IgG for 30 minutes. Finally, the sections were incubated with diaminobenzidine and hematoxylin counterstain, dehydrated in ethanol, cleared in xylene, and cover-slipped. Relative CPT-II levels were calculated *via* Image-Pro Plus 6.0.

Oil red O staining

Liver sections were generated and stained with 0.5% Oil red O solution (Jiangcheng Bioeng. Ins., Nanjing, China). The samples were subsequently observed and photographed *via* light microscopy (IX71-A12FL/PH; Olympus, Japan). The relative ratios of red lipids to the total area in the microscopic field were determined *via* Image-Pro Plus 6.0.

Biochemical detection

At baseline, every two weeks, and at sacrifice, the body weight of the mice was measured, and fasting blood samples were collected. Livers were collected after mice were sacrificed. Circulating or hepatic levels of CPT-II (Cloud-Clone Corp., United States), AFP (Finn Co., Wuhan, China), CD24 (Abcam, Shanghai, China), and CD44 (Abcam, United States) were measured *via* ELISA kits. Serum aspartate transaminase (AST), alanine transaminase (ALT), γ-glutamyl transferase (GGT), alkaline phosphatase (ALP), plasma glucose (GLU), high-density lipoprotein (HDL), low-density lipoprotein (LDL), total cholesterol (Tch), and triglyceride (TG) levels were quantitatively detected *via* routine clinical methods according to the manufacturers' instructions and standard concentration curves.

Statistical analysis

The data are expressed as the mean ± SD. All continuous variables are expressed as medians and interquartile ranges. Multiple comparisons were corrected *via* the false discovery rate (FDR-adjusted *P* value less than 0.1) method. Metabolite fold-change analysis was performed by plotting the log transformation of the *P* value obtained *via* paired Wilcoxon tests. Statistical analysis was performed *via* R software (version 4.0.4). A *P* value greater than 0.05 was considered significant.

RESULTS

CPT-II is downregulated in human HCC tissues

CPT-II at the mRNA (*CPT2*) or protein level in human HCC, non-HCC, or normal tissues is shown in Figure 1. *CPT2* is located on the IMM (Figure 1A). According to TCGA database, *CPT2* in LIHC tissues was significantly downregulated ($P < 0.001$) compared with that in normal livers (Figure 1B) or in self-controlled LIHC and non-HCC tissues (Figure 1C). Significantly lower hepatic *CPT2* mRNA levels (Figure 1D) and CPT-II protein levels (Figure 1E, top; $P < 0.01$; Figure 1E, bottom) were detected in MAFLD-related HCC and self-controlled non-HCC tissues. In addition, downregulated CPT-II levels were confirmed in the tissues of MAFLD-related HCC patients.

Hepatic pathology and LCSC markers in the MAFLD model

The dynamic alterations in the MAFLD model during hepatocarcinogenesis are shown in Figure 2. Liver appearance at different stages was associated with a HFD or HFD plus 2-FAA (Figure 2A). Livers subjected to pathological examination were divided into the NC, MAFL, MASH, LC, and HCC groups (Figure 2B). Severe lipid accumulation was observed in all groups, except for those in the NC group (Figure 2C). At liver-specific concentrations (ng/mg protein), significant CPT-II inactivity (Figure 2D) was detected, and the levels of hepatic CD44 (Figure 2E), CD24 (Figure 2F), and AFP (Figure 2G) were significantly increased with the malignant progression of hepatocytes under lipid accumulation compared to normal livers.

Blood CPT-II inactivity and LCSC activation in MAFLD

The results of the quantitative analysis of the serum biomarkers associated with the malignant progression of MAFLD are shown in Table 1. With the malignant progression of MAFLD, CPT-II and HDL were downregulated, and there was

Table 1 Circulating biochemical levels in metabolic dysfunction-associated fatty liver disease hepatocarcinogenesis

Group	NC	MAFL	MASH	LC	HCC
CPT-II (U/L)	4.69 ± 0.59	3.39 ± 0.48[a]	2.57 ± 0.36[b]	2.29 ± 0.48[b]	2.04 ± 0.29[b]
AFP (ng/L)	0.88 ± 0.09	0.96 ± 0.13	1.46 ± 0.39[b]	1.62 ± 0.42[b]	1.83 ± 0.54[b]
GLU (mmol/L)	12.04 ± 3.51	14.84 ± 3.20[a]	14.08 ± 3.20	13.21 ± 3.26	13.50 ± 4.93
ALT (IU/L)	14.75 ± 3.02	44.6 ± 14.34[b]	62.19 ± 29.79[b]	55.55 ± 26.80[b]	46.08 ± 13.39[b]
AST (IU/L)	15.88 ± 3.29	44.86 ± 9.90[b]	44.46 ± 9.25[b]	61.99 ± 18.21[b]	47.91 ± 21.22[b]
Tch (mmol/L)	1.32 ± 0.49	2.72 ± 0.46[b]	3.76 ± 0.54[b]	3.29 ± 0.24[b]	3.50 ± 0.60[b]
TG (mmol/L)	0.61 ± 0.19	0.99 ± 0.41[b]	1.78 ± 0.25[b]	1.81 ± 0.27[b]	1.22 ± 0.21[b]
HDL (mmol/L)	2.20 ± 0.42	0.67 ± 0.20[b]	1.36 ± 0.17[b]	1.17 ± 0.13[b]	1.47 ± 0.11[b]
LDL (mmol/L)	0.38 ± 0.09	1.19 ± 0.40[b]	0.65 ± 0.09[b]	0.75 ± 0.09[b]	0.63 ± 0.05[b]
CD44 (μg/L)	6.19 ± 1.88	11.43 ± 3.29[a]	16.98 ± 8.28[b]	22.63 ± 3.76[b]	30.68 ± 7.91[b]
CD24 (μg/L)	3.17 ± 0.55	9.83 ± 1.18[b]	6.50 ± 2.28[a]	9.17 ± 2.86[b]	10.50 ± 3.38[b]
ALP (U/L)	3.36 ± 2.65	5.89 ± 5.28	9.14 ± 11.1[a]	11.02 ± 12.77[b]	16.77 ± 13.08[b]
GGT (U/L)	17.16 ± 7.67	24.6 ± 9.07[a]	38.09 ± 39.35[a]	29.82 ± 24.72[a]	55.05 ± 30.64[b]

[a]$P < 0.05$, compared with the normal control (NC) group.
[b]$P < 0.01$, compared with the NC group.
AFP: Alpha-fetoprotein; ALP: Alkaline phosphatase; ALT: Alanine transaminase; AST: Aspartate transaminase; GGT: Gamma-glutamyl transferase; GLU: Glucose; HDL: High-density lipoprotein; LDL: Low-density lipoprotein; NC: Normal control; HCC: Hepatocellular carcinoma; MAFL: Metabolic dysfunction-associated fatty liver; MASH: Metabolic dysfunction-associated steato-hepatitis; LC: Liver cirrhosis; Tch: Total cholesterol; TG: Triglyceride; CPT-II: Carnitine palmitoyl transferase-II.

abnormal hepatocyte injury (ALT and AST). In addition, there were high lipid (LDL, Tch, and TG) and membrane-combined enzyme (ALP and GGT) levels with MAFLD progression. The levels of LCSC markers (CD44 and CD24) were significantly increased with the MAFLD progression, indicating that lipid accumulation results in the inactivity of circulating mitochondrial CPT-II and that LCSC activation is associated with the malignant progression of MAFLD.

T cell CPT-II and the mitochondrial membrane potential in MAFLD hepatocarcinogenesis

The dynamic alterations in CPT-II or the mitochondrial membrane potential (MMP) in T cells during MAFLD hepatocarcinogenesis are shown in Figure 3. The ratio of CD3+ T cells to peripheral blood mononuclear cells decreased with increasing age (Figure 3A). Compared with those in the NC group, the percentages of CD3+ T cells and CD4+ T cells in the liver were significantly lower in the MAFLD, MASH, LC, and HCC groups (Figure 3B). In the LC and HCC group (Figure 3C), there were increased CD8+ T cells compared to the NC group (Figure 3D). Additionally, the CPT-II concentration significantly decreased the percentage of CD4+ T cells (Figure 3F) or CD8+ T cells (Figure 3G) in the liver (Figure 3E). Moreover, the dynamic MMP (median fluorescence intensity, MFI) decreased in blood CD3+ T cells (Figure 3F), spleen CD4+ T cells (Figure 3I), and spleen CD8+ T cells (Figure 3J). Thus, these results suggested that lipid accumulation inhibits CPT-II in the IMM, reduce the MMP in intrahepatic T cells, and promote MAFLD malignant transformation.

Enrichment of DEGs in MAFLD hepatocarcinogenesis

The KEGG and GO enrichment analysis results of the DEGs in T cells are shown in Figure 4. The DEGs in IL-CD4+ cells (Figure 4A) or IL-CD8+ cells (Figure 4B) were enriched in lipid biosynthesis-related steroid, terpenoid backbone, and fatty acid pathways in the MAFL group with an immune network. The DEGs in the MASH group were enriched mainly in lipid biosynthesis-related steroids, fatty acids with glutathione, metabolism, and glycine, serine and threonine metabolism. The DEGs in the HCC group were enriched mainly in the steroid biosynthesis, PPAR, focal adhesion, ECM-receptor interaction, P53, and cell cycle pathways. Compared with those in IL-CD4+ (Figure 4C) or IL-CD8+ cells (Figure 4D), the top 10 genes were involved in energy metabolism, molecular interactions in the T cell immune system, and important signaling pathways, such as the steroid biosynthesis, cell cycle, and P53 pathways. The top 10 DEGs were enriched in T cells (Figure 4E), with 790 *vs* 183 DEGs in BB, 119 *vs* 24 DEGs in CC, 254 *vs* 116 DEGs in MF, and 28 *vs* 9 DEGs in the KEGG pathway ($P < 0.05$, Figure 4F). Thus, these findings suggested that important signaling pathways, such as the steroid synthesis, cell cycle, and TP53 pathways, are likely involved in MAFLD-related hepatocarcinogenesis.

LCSC activation in human MAFLD

An abnormal activation of circulating CD44+/CD24+ T cells in human MAFLD patients is shown in Figure 5. A lower percentage of CD44+ T cells was detected in healthy individuals (Figure 5A), whereas a higher percentage of CD44+ T cells was detected in HCC patients (Figure 5B). Similar to CD44+ T cells, circulating CD24+ T cells accounted for a lower

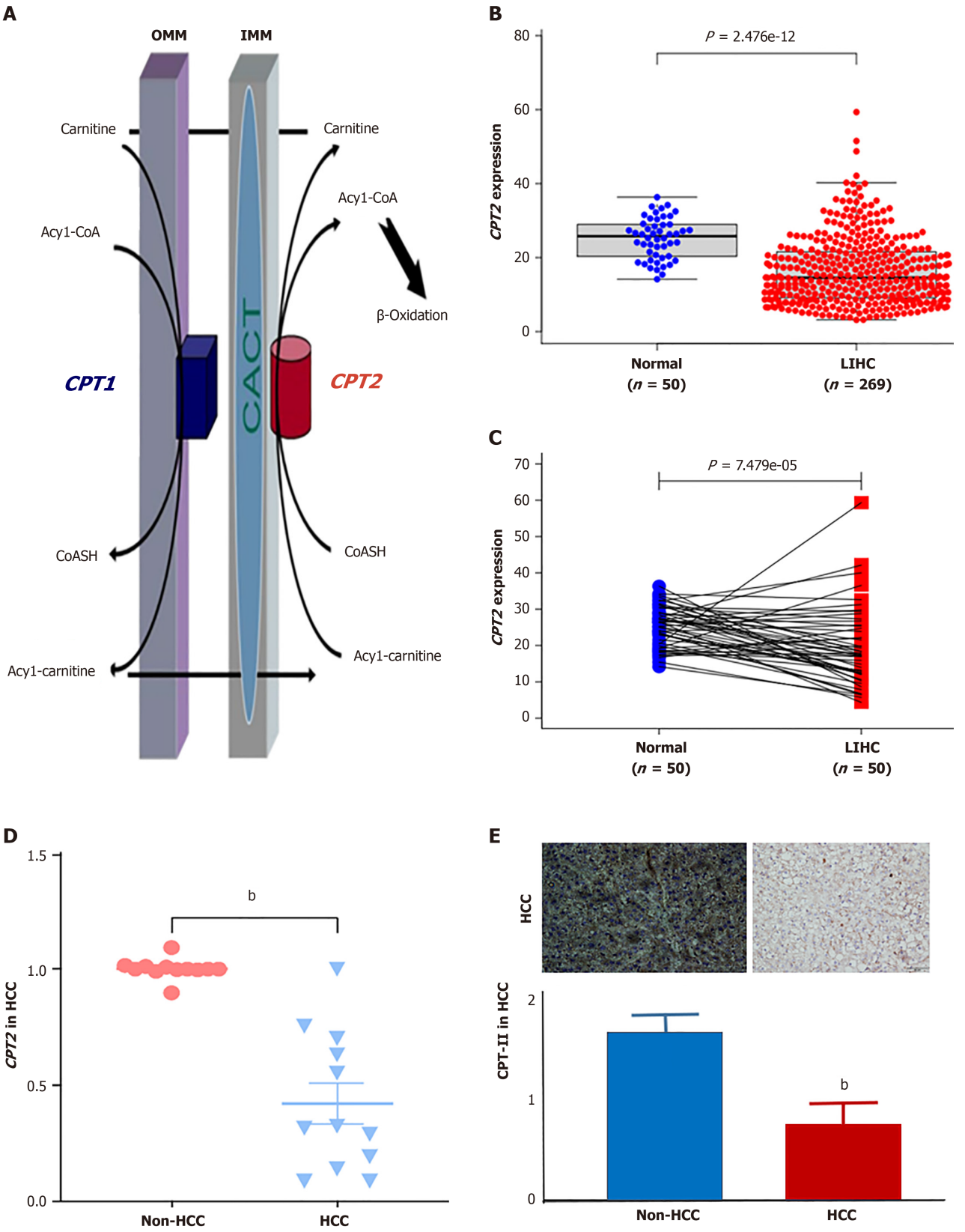


Figure 1 Carnitine palmitoyl transferase-II is downregulated in human hepatocellular carcinoma tissues. A: The carnitine palmitoyl transferase-II gene (*CPT2*) is located on the inner mitochondrial membrane; B: *CPT2* in normal liver (n = 50) or LIHC (n = 269) tissues from TCGA database; C: *CPT2* in LIHC (n = 50) and self-control normal liver (n = 50) tissues from TCGA database; D: Carnitine palmitoyl transferase-II (CPT-II) was evaluated *via* immunohistochemistry (IHC), and the relative ratio of metabolic dysfunction-associated fatty liver disease (MAFLD)-related hepatocellular carcinoma (HCC) (n = 12) to self-control non-HCC tissues (n = 12) was determined; E: CPT-II in livers: IHC (up) & specific concentration (ng/mg protein, down) between MAFLD-related HCC (n = 12) and their self-controlled non-HCC tissues (n = 12). $^{b}P < 0.01$, compared with non-HCC tissues. HCC: Hepatocellular carcinoma; IHC: Immunohistochemistry; CoA: Coenzyme A; IMM: Inner mitochondrial membrane; OMM: Outer mitochondrial membrane; *CPT2:* The carnitine palmitoyl transferase-II gene; *CPT1:* The carnitine palmitoyl transferase-I gene.

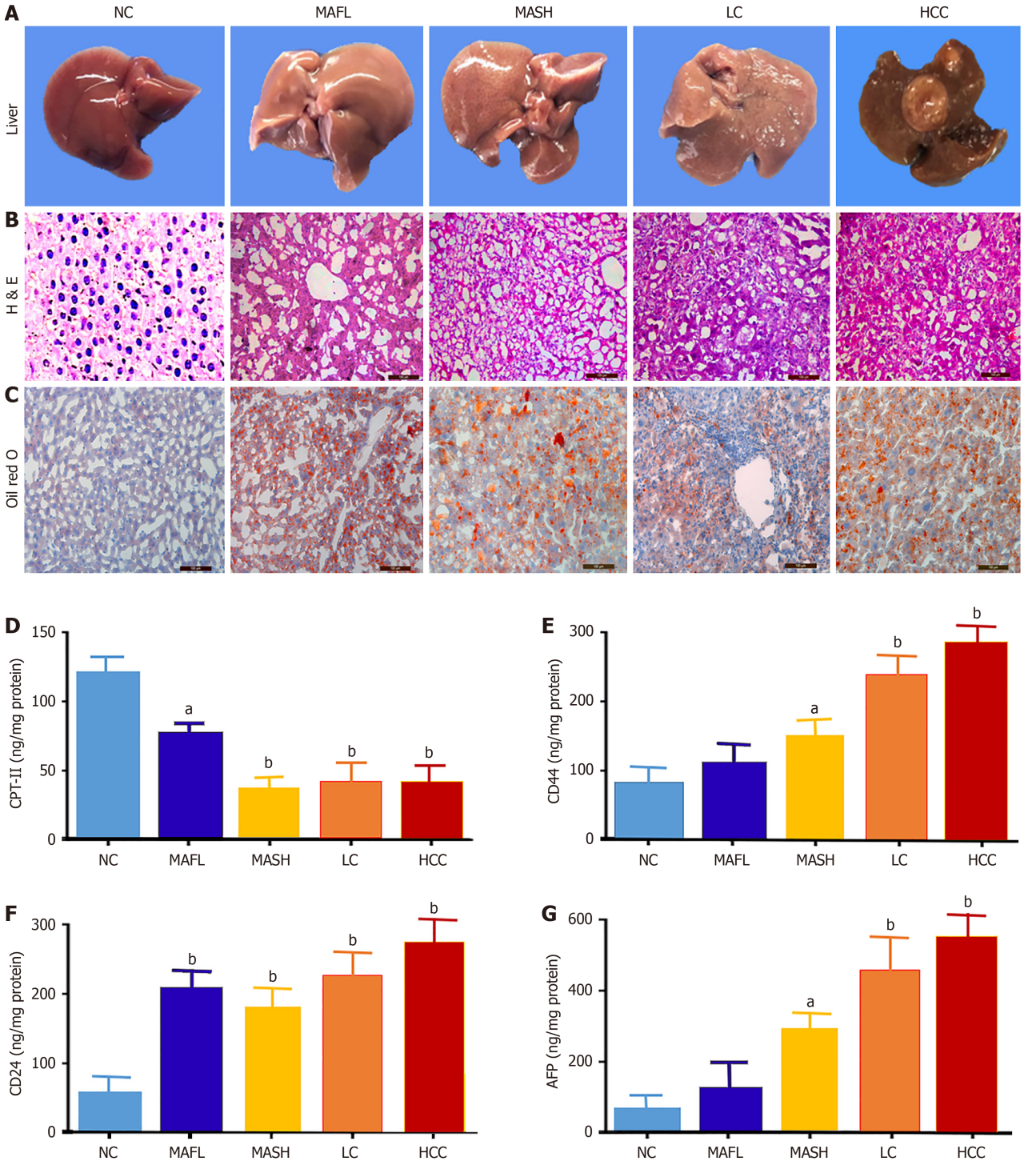
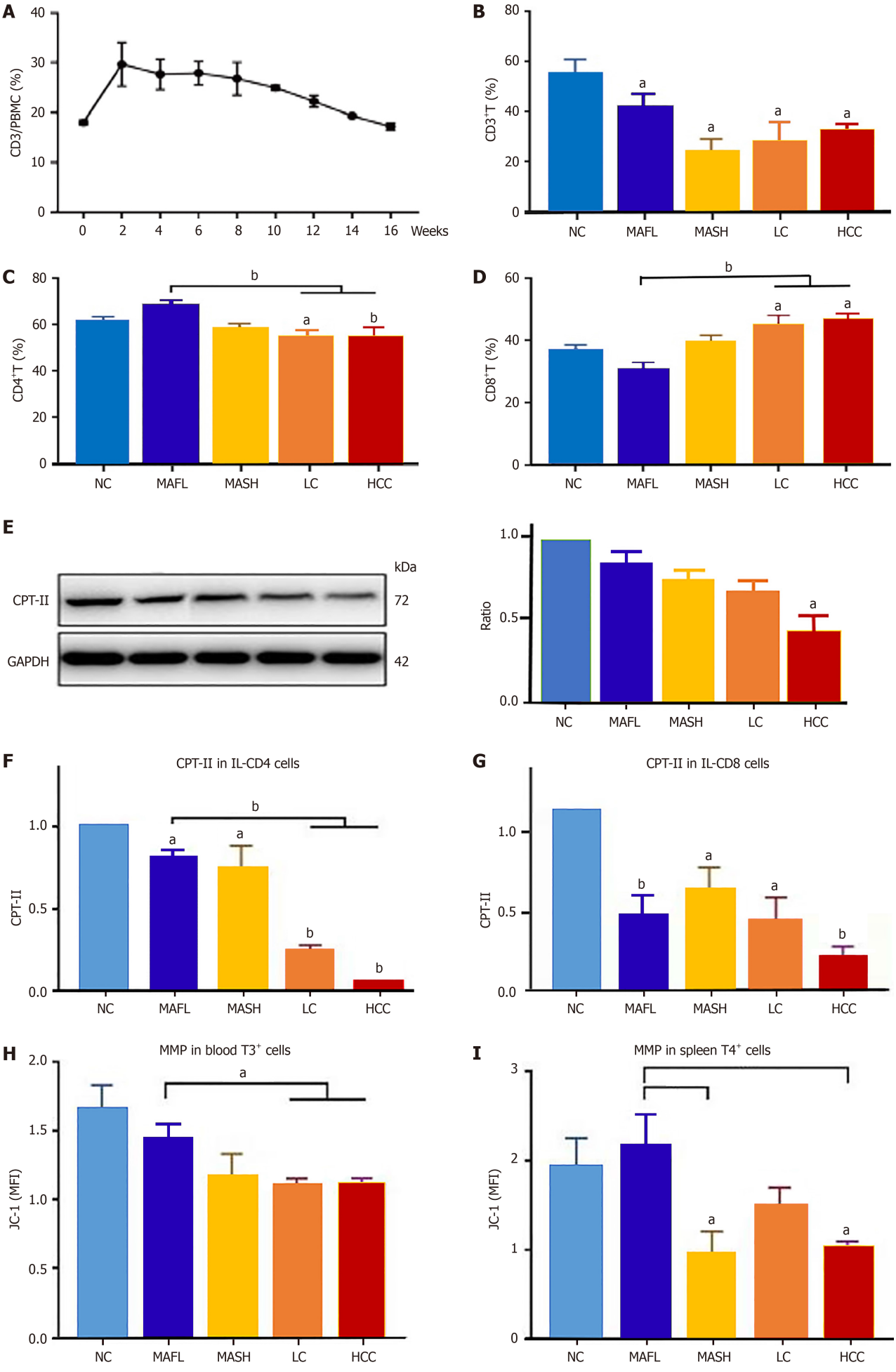


Figure 2 Metabolic dysfunction-associated fatty liver disease models, pathological alterations, and specific concentrations. A: Gross specimen of a liver; B: Liver Hematoxylin & eosin staining (100 ×); C: Oil red O staining (100 ×); D: Liver carnitine palmitoyl transferase-II specific concentration (ng/mg protein); E: Liver CD44 levels; F: Liver CD24 levels; G: Liver alpha-fetoprotein levels. $^{a}P < 0.05$, $^{b}P < 0.01$, compared with the normal control group; NC: Normal control; HCC: Hepatocellular carcinoma; MAFL: Metabolic dysfunction-associated fatty liver; MASH: Metabolic dysfunction-associated steato-hepatitis; LC: Liver cirrhosis; CPT-II: Carnitine palmitoyl transferase-II; AFP: Alpha-fetoprotein; H&E: Hematoxylin & eosin.

percentage of blood monocytes in healthy individuals (Figure 5C) and were more common in HCC patients (Figure 5D). Comparative analysis of average CD44+ T cells (Figure 5E) and CD24+ T cells (Figure 5F) among healthy individuals and MAFLD patients revealed significant differences ($P < 0.001$) between the NC group and MAFLD or HCC patients. These data suggested that a novel mechanism of mitochondrial CPT-II inactivity may promote the malignant progression of MAFLD through activated liver stem-like CD44+ or CD24+ T cells in the lipid accumulation microenvironment (Figure 5G).


A
CD3/PBMC (%)
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E
CPT-II
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Ratio
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CPT-II in IL-CD4 cells
G
CPT-II in IL-CD8 cells
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MAFL
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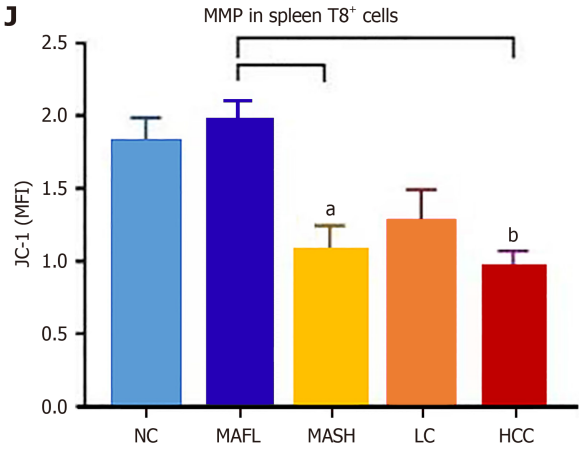


Figure 3 carnitine palmitoyl transferase-II in T cells or the mitochondrial membrane potential in metabolic dysfunction-associated fatty liver disease hepatocarcinogenesis. A: Ratios of blood CD3+ T cells to peripheral blood mononuclear cells; B: Blood CD3+ T cell percentages; C: Blood CD4+ T cell percentages; D: Blood CD8+ T cells; E: Carnitine palmitoyl transferase-II (CPT-II) was evaluated by Western blot analysis at specific concentrations; F: CPT-II in CD4+ T cells; G: CPT-II in CD8+ T cells; H: Mitochondrial membrane potential median fluorescence intensity (MFI) in blood CD3+ T cells; I: MFI in spleen CD4+ T cells; J: MFI in spleen CD8+ T cells. $^{a}P < 0.05$, compared with the normal control group; NC: Normal control; HCC: Hepatocellular carcinoma; MAFL: Metabolic dysfunction-associated fatty liver; MASH: Metabolic dysfunction-associated steato-hepatitis; LC: Liver cirrhosis; CPT-II: Carnitine palmitoyl transferase-II.

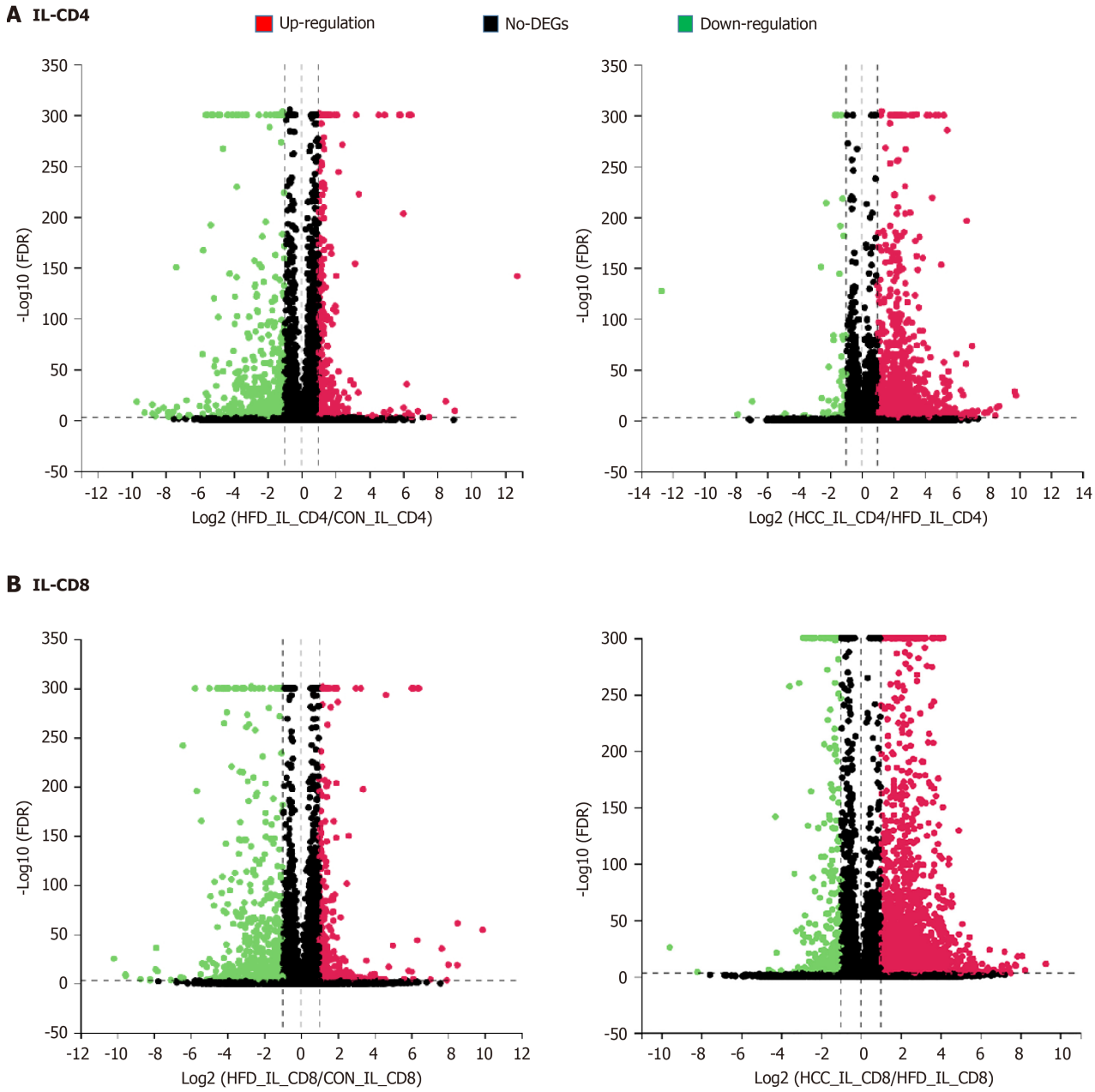
DISCUSSION

MAFLD includes a range of hepatic manifestations, starting with liver steatosis and potentially evolving toward MASH, LC, or even HCC[23-25]. Currently, the molecular pathogenesis of MAFLD is a synergistic result of mitochondrial dysfunction, lipotoxic endoplasmic reticulum stress, and several immune cell-mediated inflammatory processes on the basis of steatosis and lipid oxidation[26,27], particularly in MASH, at which point inflammation becomes integral to disease progression[28]. Previous studies have reported CPT-II inactivity in MAFLD patients[21,29]. Similar trends have been confirmed in the tissues of HCC patients from TCGA database or in patients whose CPT-II concentrations are downregulated in HCC. However, the exact relationship between CPT-II and MAFLD progression remains to be explored. The present study investigated CPT-II inactivity with respect to LCSC activation and T cell antitumor function.

Abnormal lipid metabolism and mitochondrial damage are associated with MAFLD progression[30]. Mitochondria are the major sites of β-oxidation, a catabolic process by which fatty acids are broken down[31]. Excessive lipid accumulation and uncontrolled production of ROS, including membranes, proteins, and mtDNA, can damage mitochondria[32] and trigger the mitochondrial pathway of apoptosis. In addition, MMP stability is conducive to maintaining mitochondrial function[33,34]. In the present study, downregulated CPT-II and the MMP in the IMM were confirmed to adapt to a fat-rich environment. The average levels of CPT-II and MMP in the LC and HCC groups were significantly lower than those in the MAFLD group. A decrease in *CPT2* and the MMP in T cells may significantly affect MAFLD progression. These data suggested that alterations in CPT-II or the MMP in the IMM may be early monitoring markers for the malignant progression of MAFLD[35,36].

In MAFLD, a tumor-prone liver microenvironment exists, in which both the innate and adaptive immune systems are involved[37]. Lymphocyte interactions play important roles in MAFLD progression. T cells are the primary target cells for HCC immunotherapy, mainly *via* reactivation of the immune system, including immune checkpoint inhibitors, multifunctional antibodies, and adoptive cell therapy[38]. As the most typical antitumor effector, T cell immunological functions are remodeled by chronic inflammation, metabolic alterations, lipid toxicity, and oxidative stress from MASH to HCC[39]. In the present study, abnormal lipid metabolism during the course of MAFLD inevitably decreased the number of IL-CD3^{+} and IL-CD4^{+} T cells *in vivo* by activating immune cells to produce responses, such as reprogramming lipid metabolism, increasing lipid synthesis, and increasing cholesterol uptake. These data indicate that hepatic *CPT2* regulates mitochondrial function and depletes IL-CD3+ and IL-CD4+ T cells in the adaptive immune response[19,40].

Lipid metabolism disorders in MAFLD directly cause chronic inflammatory responses *via* the production of LCSCs, which typically express the CD44, CD47, CD24, and CD133 biomarkers[21,41]. In the clinic, the levels of circulating CD24 and CD44 in HCC patients are significantly greater than those in healthy controls or MAFLD patients[42,43]. LCSCs constitute a small fraction of cancer cells with stem cell properties, and they play important roles in self-renewal, growth, metastasis, recurrence, and drug resistance in HCC[44]. LCSCs may interact with immune cells, alter or impair the natural function of immune cells, and participate in the formation of an immunosuppressive microenvironment to evade immune surveillance, which are key factors contributing to the poor prognosis of HCC patients[45]. In the present study, the percentage of circulating CD44+ or CD24+ T cells was investigated in MAFLD patients and healthy individuals with a low ratio of CD44+ or CD24+ T cells and in HCC patients and MAFLD patients with a high ratio of CD44+ or CD24+ T cells. Significant differences were found between the NC group and the MAFLD or HCC group. A novel mechanism of CPT-II inactivity may promote the malignant progression of MAFLD *via* the activation of LCSCs in the lipid accumulation microenvironment.


A IL-CD4
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Log2 (HCC_IL_CD4/HFD_IL_CD4)
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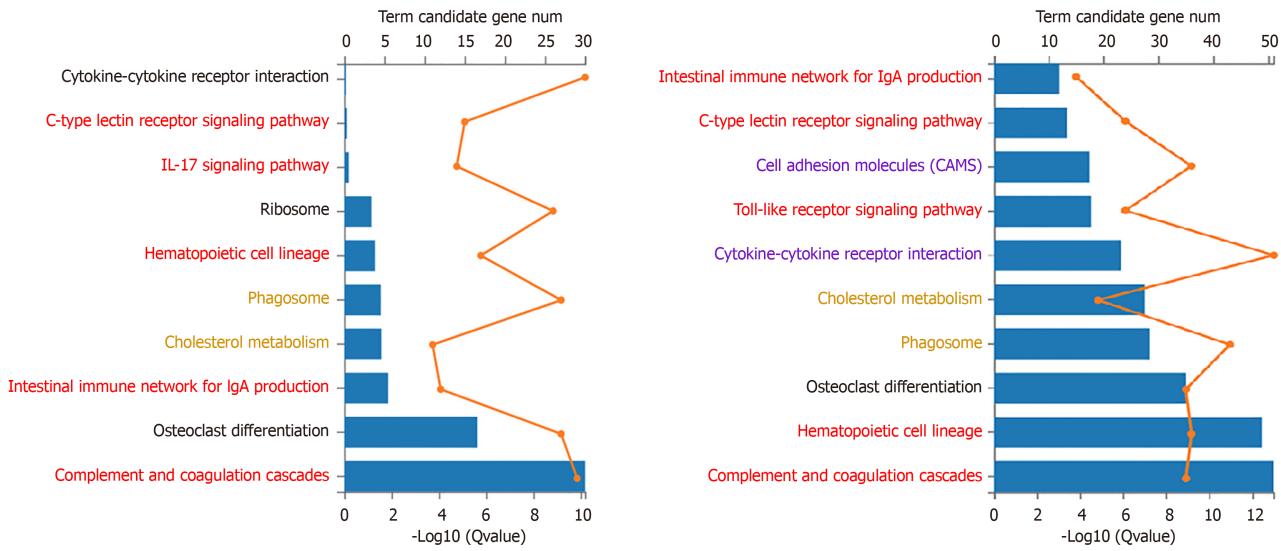
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Intestinal immune network for IgA production
Osteoclast differentiation
Complement and coagulation cascades
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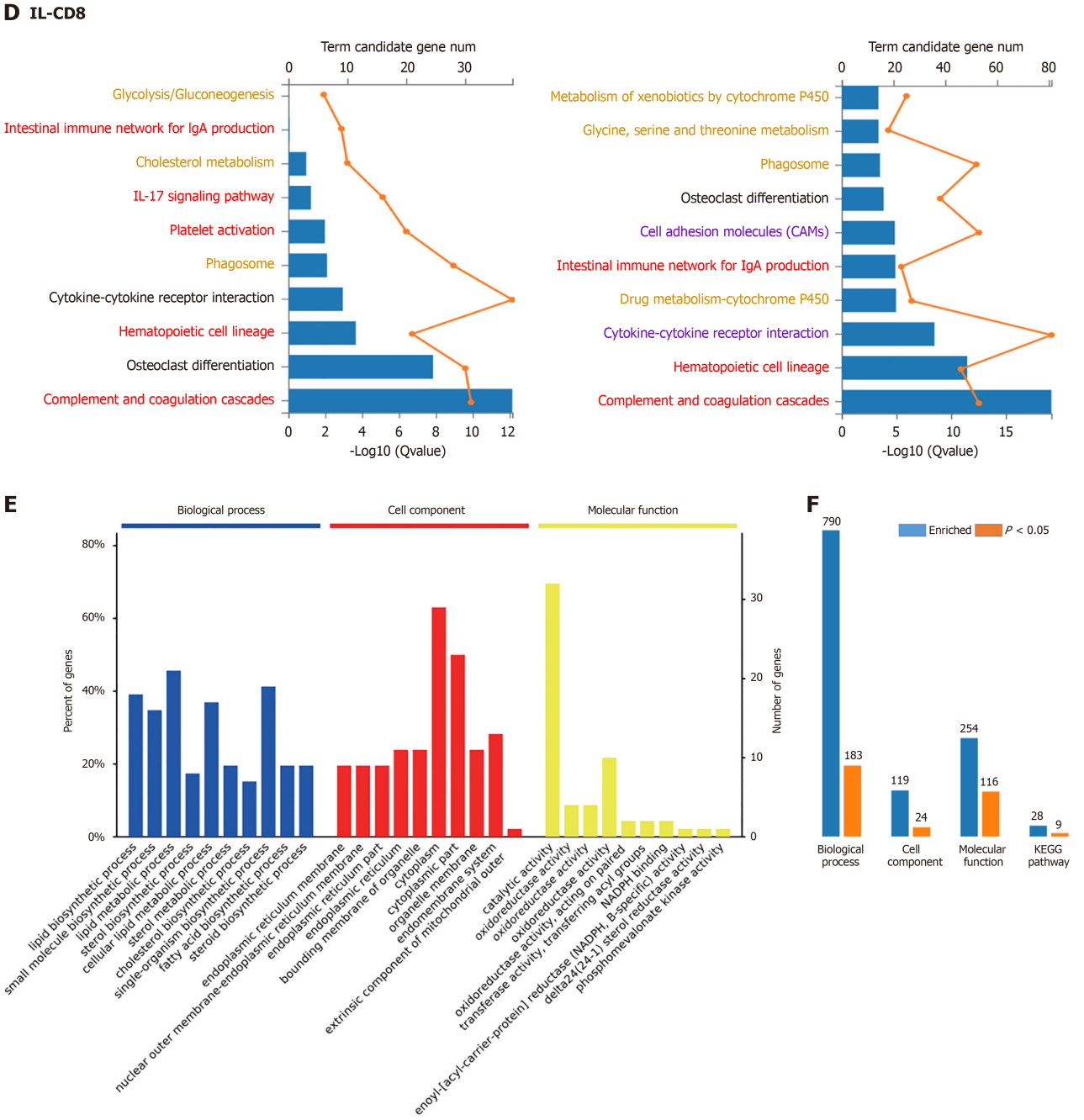

Figure 4 Enrichment analysis of T cell differentially expressed genes in metabolic dysfunction-associated fatty liver disease hepatocarcinogenesis. A: Differentially expressed genes (DEGs) in IL-CD4+ T cells. Left, metabolic dysfunction-associated fatty liver (MAFL) *vs* normal control (NC); Right, hepatocellular carcinoma (HCC) *vs* metabolic dysfunction-associated steatohepatitis (MASH); B: DEGs in IL-CD8+ T cells. Left, MAFL *vs* NC; Right, HCC *vs* MASH; C: Top 10 DEGs in IL-CD4+ T cells. Left, MAFL *vs* NC; Right, HCC *vs* MASHc; D: Top 10 DEGs in IL-CD8+ T cells. Left, MAFL *vs* NC; Right, HCC *vs* MASH; E: Gene Ontology (GO) analysis of DEGs; F: KEGG pathway with distribution of the corresponding GO terms of the top 332 features in hepatocarcinogenesis. $^{a}P < 0.05$, compared with the NC group; NC: Normal control.

Growing evidence shows that MASH is likely to facilitate the transition of T cells in terms of cell motility, effector function, metabolic reprogramming, and gene transcription[46,47]. Immune therapy for nonviral HCC does not improve survival in patients. MASH-HCC may be less responsive to immunotherapy because MASH-related aberrant T cell activation causes tissue damage that leads to impaired immune surveillance[37,48]. In the present study, many DEGs whose expression was altered at the IL-CD4+ or IL-CD8+ T cell transcriptional level were enriched mainly in energy metabolism and molecular interactions in IL T cells under the dynamic expression of CPT-II. Bioinformatics analysis revealed that the DEGs in MAFLD were associated with the lipid biosynthesis-related steroid, terpenoid backbone, and fatty acid pathways in the immune network. However, the DEGs associated with the malignant progression of MAFLD were enriched mainly in the PPAR, focal adhesion, and ECM-receptor interaction pathways and related signaling pathways, such as the steroid biosynthesis, cell cycle, and P53 pathways. These findings suggested that MASH-related aberrant T cell activation, especially LCSC activity or restoring CPT-II activity, may be considered for immunotherapy[49,50].

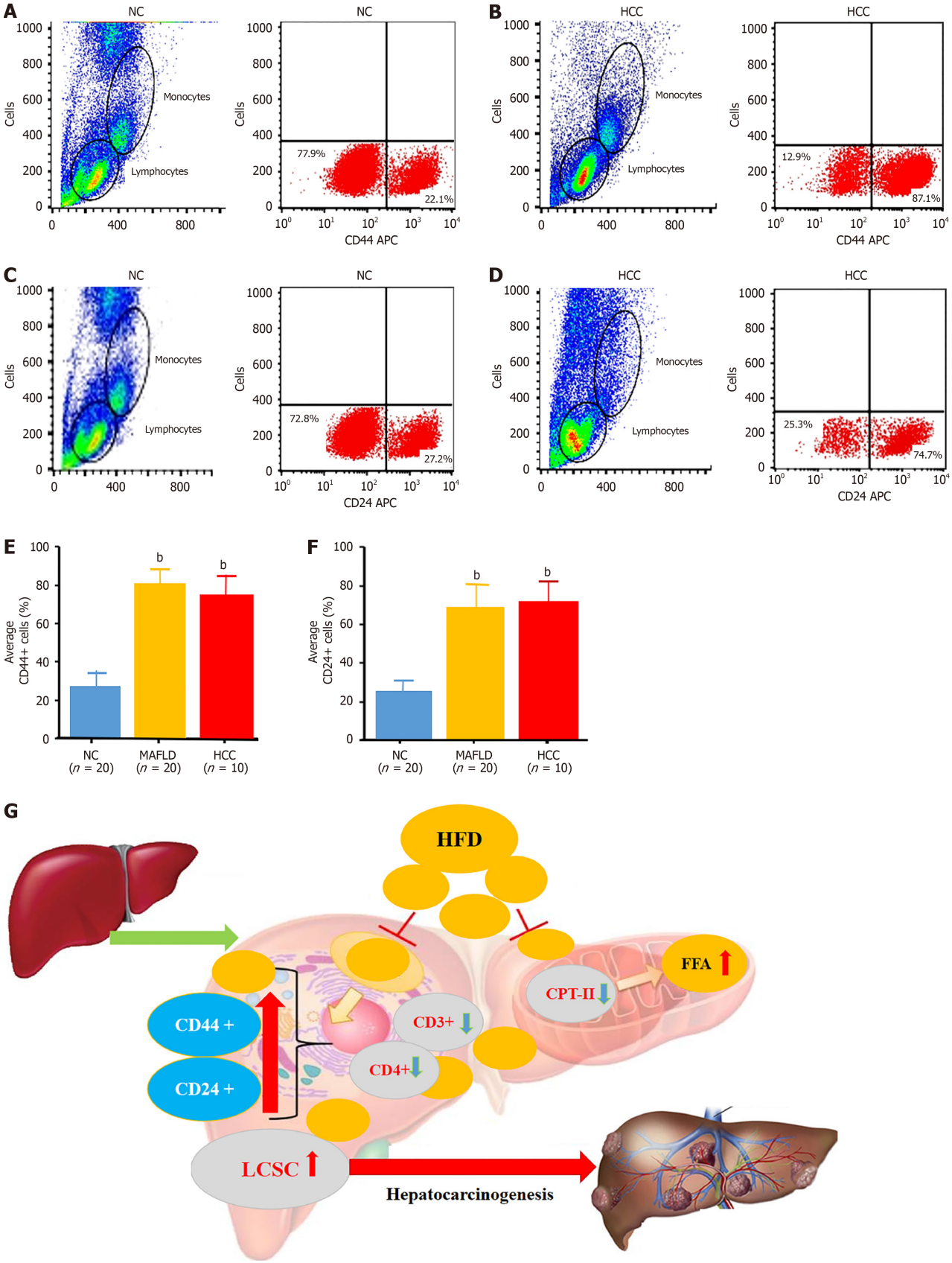

Figure 5 Validation of liver cancer stem cell activation in human metabolic dysfunction-associated fatty liver disease. A: Blood monocytes and CD44+ cells in healthy individuals; B: Blood monocytes and CD44+ cells from hepatocellular carcinoma (HCC) patients; C: Blood monocytes and CD24+ cells in healthy individuals; D: Blood monocytes and CD24+ cells in HCC patients; E: Average number of CD44+ cells in the blood of metabolic dysfunction-associated fatty liver disease (MAFLD) or HCC patients; F: Average number of CD24+ blood cells in MAFLD and HCC patients; G: A possible mechanism of hepatic carnitine palmitoyl transferase-II inactivity in MAFLD-related hepatocarcinogenesis involves the activation of LCSCs. $^{b}P < 0.01$, compared with the normal control; LCSC: Liver cancer stem cell; MAFLD: Metabolic dysfunction-associated fatty liver disease; HFD: High-fat diet; NC: Normal control.

On the basis of these findings, the dynamic alterations in mitochondrial CPT-II in T cells were investigated, and LCSC activation in MAFLD was first investigated during the progression of hepatocyte malignancy. Lipid accumulation resulted in a decrease in mitochondrial CPT-II activity and the MMP with decreasing numbers of hepatic CD3+ or CD4+ T cells. These discoveries were validated in the tissues or blood of MAFLD-related HCC patients. Mechanistically, CPT-II inactivity *via* the loss of innate immune function with abnormal activation of LCSCs promotes the malignant progression of MAFLD. However, the present study had several limitations. First, the sample size was small, and the study lacked an external validation cohort. In addition, because the present study involved model discovery, a limited number of clinical samples were validated. The consideration of a more comprehensive multicenter sample may confirm this possible new mechanism. CPT-II may prevent lipid accumulation or LCSC activation, as well as improve IL-CD3/CD4 T cell function for HCC immunotherapy and delay the malignant progression of MAFLD.

CONCLUSION

Hepatic CPT-II inactivity promotes the malignant progression of MAFLD *via* the loss of innate immune function and abnormal LCSC activation.

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FOOTNOTES

Author contributions: Wang LL, Lu YM, and Wang YH conceptualized and designed the research; Wang YH, Wang YF, and Fang RF screened patients and acquired clinical data; Yang YF and Sai WL collected blood specimens and performed laboratory analysis; Fang RF and Sai WL performed data analysis; Yao DF and Yao M acquired the funding and wrote the manuscript. All the authors have read and approved the final manuscript. Wang LL, Lu YM, and Wang YH were responsible for patient screening, enrollment, and collection of clinical data and blood samples. Both authors have made crucial and indispensable contributions toward the completion of the project and thus have qualified as the co-first authors of the paper. Both Yao M and Yao DF played important and indispensable roles in the experimental design, data interpretation, and manuscript preparation as co-corresponding authors. Yao M and Yao DF applied for and obtained the funds for this research project, as well as conceptualized, designed, and supervised the entire process of the project. Yao M and Yao DF searched the literature and revised and submitted the early version of the manuscript with a focus on the association between MAFLD and LCSCs, and they were instrumental and responsible for data reanalysis and reinterpretation, figure plotting, comprehensive literature search, preparation and submission of the current version of the manuscript, with a new focus on LCSCs as predictors of MAFLD and potential underlying mechanisms. This collaboration between Yao M and Yao DF is crucial for the publication of this manuscript and other manuscripts still in preparation.

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