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Research article

ELOVL1 is upregulated and promotes tumor growth in hepatocellular carcinoma through regulating PI3K-AKT-mTOR signaling

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ABSTRACT

Background: The functions of the ELOVLs are mainly involved in the elongation of saturated and polyunsaturated fatty acids, thus influencing the metabolism of fatty acids. Abnormal lipid metabolism may result in NAFLD and NASH, which may lead to cirrhosis and liver cancer. These results suggest that ELOVLs-mediated metabolism might be involved in the development of HCC. The purpose of this study was to study the expression and function of ELOVL1 in human liver cancer.

Method: Using TCGA, GEPIA and other databases, we analyzed the relationship between the expression of ELOVL1 and liver cancer. The expression of ELOVL1 was detected by immunohistochemical method and Western blot method in hepatic carcinoma and hepatic carcinoma cells. Then, the effects of ELOVL1 on proliferation, apoptosis and invasion in vitro and in vivo were investigated by means of different methods.

Result: Our results indicate that ELOVL1 is more highly expressed in liver cancer than in normal tissues. Survival analysis showed that OS and DSS were shorter in patients with high ELOVL1 expression than in those with low expression. Multivariate Cox analysis further demonstrated that over-expression of ELOVL1 was an independent risk factor for overall survival in HCC. The results of ROC also confirmed the value of ELOVL1 in the diagnosis of liver cancer. The results of KEGG enrichment and GSEA indicate that ELOVL1 is associated with lipid metabolism and NAFLD, as well as PPAR, PI3K-AKT-mTOR. Compared with the control group, it was found that silencing ELOVL1 in Huh7 and HepG2 cells could inhibit the growth of cells, promote the apoptosis and decrease the metastasis and invasion. Changes in ELOVL1 induced cell proliferation and metastasis may be related to PI3K/AKT/mTOR. Low expression of ELOVL1 inhibited the growth of xenograft tumors in hepatocellular carcinoma xenograft model.

Conclusion: Our data indicate that the activation of PI3K/AKT/mTOR pathway in HCC may contribute to the promotion of cancer. Thus, ELOVL1 may be a promising therapeutic target for HCC.

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1. Background

HCC is a high degree of malignancy, and its prognosis is poor, accounting for about 90 % of primary hepatic carcinoma. It is the most prevalent malignant liver disease and its incidence is increasing globally, making it the fourth leading cause of cancer-related deaths worldwide [1,2]. HCC usually occurs in cirrhosis of the liver due to chronic liver disease. Common risk factors are hepatitis B (HBV) and HCV (HCV), alcohol abuse, metabolic hepatic impairment (e.g., non-alcoholic fatty liver), and long-term exposure to food toxins like aflatoxin and aristolochic acid [3,4]. In China, the prevalence of hepatitis B virus infection is relatively high, with a large number of infected individuals, leading to a higher incidence of HCC compared to Western countries [5]. Early-stage HCC patients are treated with surgical resection, liver transplantation, and local ablation [6]. However, the early signs of HCC are usually not obvious, leading to the diagnosis in the middle or late stages, resulting in a high recurrence rate [7]. As a result, patients are limited to non-surgical treatments such as chemotherapy or radiotherapy. Unfortunately, cancer cells often exhibit low sensitivity to these treatments, leading to unsatisfactory therapeutic outcomes and a lack of effective treatment options for patients [8]. It is essential to explore the mechanisms underlying HCC's development and development, and to identify new strategies for effective suppression of its growth and metastasis.

Lipids are a complex and diverse group of molecules, mainly including fatty acids (FAs), cholesterol and phospholipids (PLs). Lipid molecules are involved in most physiological processes of human body [9,10]. Hepatic lipid metabolism is controlled by fatty acid intake and output, the production of new products and the utilization of β oxidation [11]. When the balance between the above pathways is broken, lipids begin to accumulate in the liver, resulting in nonalcoholic fatty liver disease (NAFLD) [12]. NAFLD is a major manifestation of hepatic metabolic syndrome, which includes extensive liver damage ranging from simple steatosis to severe nonalcoholic steatohepatitis (NASH), and finally to cirrhosis and HCC [13]. The composition of long-chain fatty acids in the liver is a new factor for NAFLD, and NAFLD may be a risk factor for HCC, it is considered that lipids can promote the occurrence of hepatocellular carcinoma.

ELOVLs are the first rate-limiting enzyme for the VLCFA synthesis, which controls the rate and direction of this metabolism. So far, 7 genes have been identified, including ELOVL1-7 [14,15]. Research has demonstrated the involvement of ELOVLs family proteins in X-linked adrenoleukodystrophy (X-ALD) [16], ichthyosis [17], spinocerebellar ataxia type 34 (SCA34) [18] and other diseases. Several genes in this family have been identified as significantly upregulated or downregulated during the development of breast, kidney, and prostate cancers [19]. High expression of ELOVL1 in colorectal carcinoma (CRC) and breast carcinoma may influence the development of cancer and be related to the malignancy. However, ELOVL1's clinical significance and role in HCC remains unclear. Thus, a better characterization of these proteins may provide new insights into the treatment of HCC.

It is known that PI3K/AKT/mTOR pathway plays an important role in cell growth, proliferation, differentiation, migration, survival and cell migration [20]. PI3K is the initiator of PI3K/AKT/mTOR signaling pathway, and AKT and mTOR are important downstream targets. Many of the PI3K/AKT/mTOR genes are unbalanced in the course of cancer progression, which is responsible for the death and proliferation of cancer cells [21]. PI3K/AKT/mTOR also plays an important role in lipid metabolism. Sterol regulatory element binding protein −1 (SREBP-1) is a key regulator of genes related to the synthesis of liver triglyceride (TG) and plays an important role in the development of fatty liver. Activation of PI3K/AKT pathway can enhance the activity of SREBP-1, so PI3K/AKT activation may induce the occurrence and development of fatty liver [22]. Moreover, there is a close relationship between lipid metabolism and autophagy. PI3K/AKT/mTOR is the classic mechanism of autophagy regulation, and mTOR plays an important role in autophagy [20]. Studies on hepatocellular carcinoma show that activating AKT/mTOR pathway can increase the expression of fatty acid oxidation (FAO) regulator SREBP1c, and then reprogram liver lipid metabolism [23].In conclusion, the PI3K/AKT/mTOR pathway plays a key role in lipid metabolism, which suggests that ELOVL1 might regulate PI3K/AKT/mTOR and promote the development of liver cancer.

In this paper, we studied the relationship between the expression of ELOVL1 and the clinical pathology of HCC. Subsequently, in vitro and in vivo tests were carried out to study the effect of ELOVL1 on the growth and metastasis of HCC cells, and explored of its mechanism.

2. Methods

2.1. Bioinformatics analysis

Retrieval of ELOVL1 gene expression data from TCGA database (Tumor Genome Atlas) in HCC (https://www.cancer.gov/ccg/research/genome-sequencing/tcga). [24]. We utilized R language software (version 4.2.1) and its corresponding packages for data processing to extract pertinent clinical information and elucidate the functional role of ELOVL1 [25]. Log in to GEO database, find the data sets related to hepatocellular carcinoma, and use R language software to sort out and analyze the data to verify whether the expression of ELOVL1 in hepatocellular carcinoma and adjacent tissues is consistent with TCGA database. We used the GEPIA database (http://gepia.cancer-pku.cn/index.html) to analyze the overall survival and disease-free survival of the ELOVL1 gene and the 50 % threshold for differentiating high and low expression groups [26]. Access to the Human Protein Atlas (Human Protein Atlas) immunohistochemical database (https://www.proteinatlas.org/) to obtain ELOVL1 immunohistochemical results in both normal hepatic and hepatic cancer tissues [27].

2.2. KEGG pathway enrichment analysis and GSEA for ELOVL1

On the basis of the correlation of all the genes and ELOVL1 expression, it was found that the genes with up and down regulation >1 and Padj <0.05 were classified as differential expression genes. Then KEGG and GSEA are used to create a sequence of genes, and the R language is used to sort out the data and do visual analysis.KEGG, as a database, can analyze genomics, biology, disease, medicine and chemicals [28]. GSEA is a gene in a predefined gene set to evaluate its distribution in the gene table ranked by phenotypic correlation, so that we can understand the contribution of related genes to phenotype.The predefined set of genes comes from the MSigDB dataset (https://www.gsea-msigdb.org/gsea/msigdb/index.jsp). On the basis of the association of all the genes with the expression of ELOVL1, GSEA was used in our study to get an ordered list of genes. Based on the nominal P value and the normalized enrichment score (NES), the pathways associated with enrichment and phenotype were determined [29].

2.3. Tissue samples collection and cell culture

The Ethics Committee of Guizhou Medical University's Affiliated Hospital approved the study, and informed consent was obtained from every patient. The tumor tissues of Guizhou Medical University Affiliated Hospital were collected and diagnosed as HCC by pathology. ELOVL1 immunohistochemistry was used to detect the collected tumor samples and their paired adjacent tissues. We purchased two human hepatocellular carcinoma cell lines, Huh7 and HepG2(BNCC, source:human), as well as the normal hepatocyte line LO2(BNCC, source:human), Cultured in modified Eagle medium (DMEM, Hyclone), 10 % fetal bovine serum (Gibco) and 1 % penicillin-streptomycin (Corning) were added. The cells were cultured at 37 °C with 5 % CO_2.

2.4. Immunohistochemical staining

The peroxidase coupling method was utilized to detect ELOVL1 expression in tissues. Briefly, the paraffin-embedded tissue fixed by paraformaldehyde was cut into 5um slices and treated with 3 % hydrogen peroxide made of 30 % hydrogen peroxide and methanol in a ratio of 1: 4 for 10min. Sheep serum was dropped onto the slices for 25 min to block them, followed by rinsing with PBS. ELOVL1 polyclonal antibody was added to the slices and incubated at room temperature for 1 h. They were incubated for 30 min with goat anti-rabbit IgG (ab205718, 1: 5000, Abcam) coupled with horseradish peroxidase (HRP), followed by incubation with streptavid-horseradish peroxidase complex. After washing and visualization of the glass slide, the substrate diaminobenzidine tetrachlorohydrochloride was added, and then dyed with hematoxylin. Finally, the film is sealed, photographed and observed.

2.5. Transduction of sh-ELOVL1 interfering lentivirus

We designed three RNA interference (RNAi) sequences targeting the ELOVL1 gene. The interference sequences were synthesized as double-stranded DNA oligos, cut by enzymes at both ends, and directly ligated into the pGMLV-SC5 RNAi vector (Shanghai JiMan Science and Technology Co., Ltd.). This generated H_ELOVL1-shRNA507 (PGMLV-ZsGreen-Puro), H_ELOVL1-shRNA680 (PGMLV-ZsGreen-Puro), and H_ELOVL1-shRNA567 (PGMLV-ZsGreen-Puro). The RNAi vector contains markers for fluorescent protein tracking and resistance selection. Empty lentivirus was used as the negative control group, while recombinant interference lentivirus carrying ELOVL1 and empty lentivirus were separately transfected into Huh7 and HepG2 cells. After 48 or 72 h of transfection, the cells which were successfully transfected were selected with puromycin, and the fluorescent signals were observed under fluorescent microscopy. Finally, qRT-PCR and Western blotting were used to confirm the stability of the transfected HCC cells. Then, we select the cells with the highest interference efficiency for the following experiments.

2.6. Real-time quantitative RT-PCR

Total RNA was extracted with TRIzol (Invitrogen), and the total RNA concentration was measured by NanoDrop. Using FastKing RT Kit (With gDNase) FastKing cDNA First Strand Synthesis Kit (Invitrogen), the first strand of cDNA was synthesized. Then, Taq Pro Universal SYBR qPCR Master Mix (Invitrogen) was used to amplify the target gene. The q-PCR primer sequences are as follows: GAPDH forward 5′-TTGCCCTCAACGACCACTTT-3′ and reverse 5′-TGGTCCAGGGGTCTTACTCC-3'; ELOVL1 forward 5′-CCAGTCATTATT-CACCTCAT-3′ and reverse 5′-AAGAGTGATACCAGAAGTTG-3'. Data analysis was performed using the comparative $2^{-\Delta\Delta Ct}$ method.

2.7. Western blotting

The total protein was extracted with a RIPA lysis buffer. The BCA assay kit (Thermo Fisher Scientific, Inc.) was used to measure the protein concentration. The SDS-PAGE was separated and transferred to the PVDF membrane. The PVDF membrane was sealed with 5 % skim milk and was incubated with ELOVL1, p-PI3K, *p*-Akt and *p*-mTOR at 4 °C overnight. Incubation was carried out for 1 h with the second antibody. Finally, BioImaging Systems was used to visualize the bands.

2.8. Apoptosis and cell cycle analysis

We used the PI/Annexin V-FITC staining kit to detect cell death and PI staining to determine the cell cycle. After trypsin digestion, cells were collected for apoptosis and cycle detection. The cells were fixed at 4 °C in 70 % alcohol for 2 h or overnight for cell cycle

analysis. The relevant data were obtained by means of flow cytometric analysis using PI, Annexin V-FITC, or PI itself as instructed. The data were analyzed using FlowJo software.

2.9. Cell proliferation assay

The Cell Counting Kit-8 (CCK-8) was used in this study to evaluate cell viability. Specifically, 3×10 cells were seeded into each well of a 96 well plate and cultivated for a predetermined time. The optical density of each well was then measured at 450 nm with a microplate reader. In certain experiments, the mTOR inhibitor rapamycin was introduced to the cultured cells.

2.10. Cell cloning experiment

After digesting and collecting cells with trypsin for cell counting under a microscope, 1×10^3 cells were seeded into each well of a 6-well plate. Cells were observed and the medium was replaced every 3–4 days. After 14 days of cultivation, there were visible clone spheres. They were fixed with polyformaldehyde and stained with crystalline violet. The number of clones was counted and analyzed under a microscope.

2.11. Scratch test

The cells were seeded in a 6-well plate and cultured until they reached approximately 90 % confluency. A pipette tip was then used to create a scratch in the middle of the long and short axes of each well. The detached cells were removed, and complete culture medium was added for further cultivation. The healing of the cell scratch was monitored by taking photographs at different time points, and the percentage of the healed area was calculated based on each photo.

2.12. Invasion experiment

Place the Transwell chamber into a 24-well plate, and then add Matrigel to the Transwell chamber and spread it evenly. Digest the cells with pancreatic enzymes, count them, and add $1X10^4$ cells into the Transwell-Matrigel chamber. Incubate for 48 h. Remove the Transwell chamber and fix it in a solution of polyformaldehyde. After fixation, remove the Matrigel and stain with crystal violet solution. Observe and analyze under a microscope.

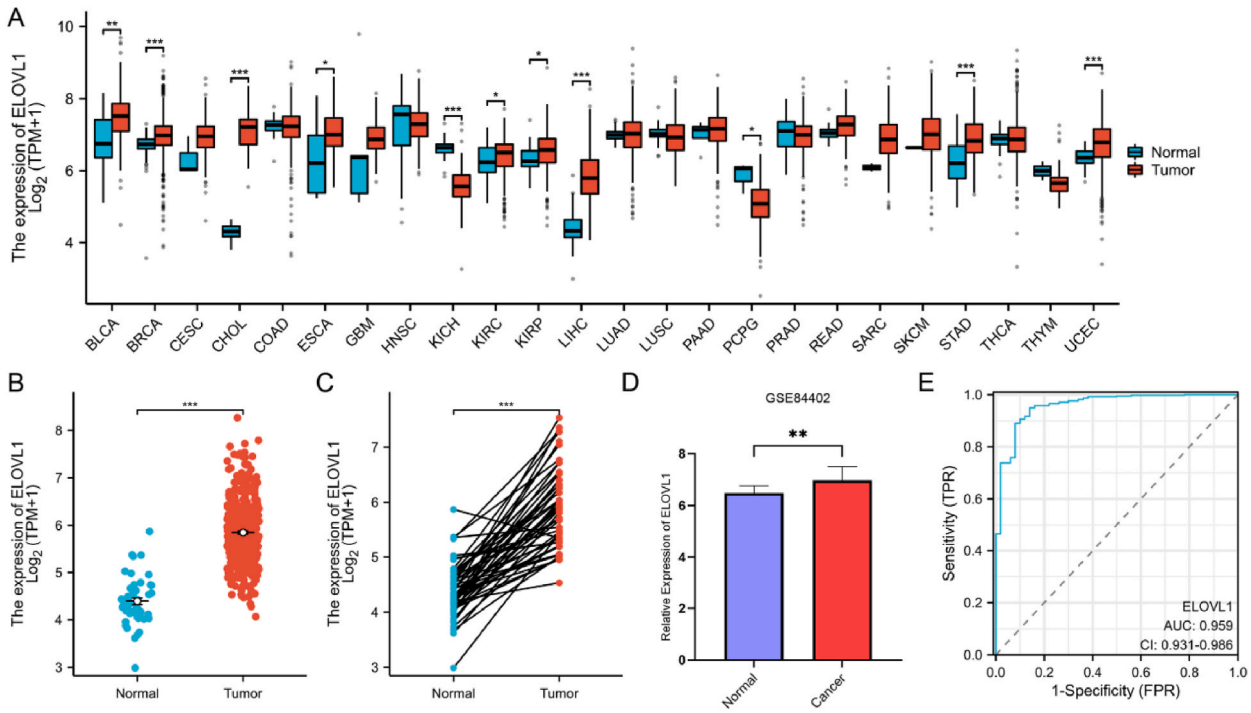

Fig. 1. Expression of ELOVL1 in Pan-Cancer and hepatocellular carcinoma. (A) The differential expression of ELOVL1 in various tumor tissues and normal tissues; (B) The relative expression level of ELOVL1 in hepatocellular carcinoma tissues and normal tissues; (C) The relative expression level of ELOVL1 in hepatocellular carcinoma tissues and paired adjacent non-cancerous tissues; (D) Verification of ELOVL1 expression in hepatocellular carcinoma by GEO database; (E) Diagnostic ROC curve.

2.13. Subcutaneous tumor formation experiment in nude mice

Huh7 cells were transfected with lentivirus to construct stable transfected cell lines for future use. Select female nude mice aged 6–8 weeks(Beyotime Biotech lnc), and cultivate them in a SPF-level environment for 2 weeks to adapt to the culture conditions, ensuring that the nude mice are in a good state for subsequent experimental operations. The mice were randomly divided into 3 groups, each of which was made up of 5 mice. Adjust the concentration of the transfected backup cells to 1 × 107 cells/ml using PBS. Inoculate 0.2 ml of cell suspension under the armpit of each nude mouse. Approximately 5–7 days later, tumor nodules will form, and then the tumor volume will be measured every 3 days (Volume = (long diameter × short diameter^2)/2). 21 days later, the mice were killed by the method of cervical dislocation. Careful removal of the subcutaneous tumors was performed. Then the body weight was measured to plot the growth curve. Finally, the tumor mass, the liver, and the lungs were taken. The tissues were separated into two parts: one was kept in a refrigerator of −80 °C, and the other was kept in 4 % paraformaldehyde.

2.14. Statistical analysis

A GraphPad Prism 8.0 was used for statistical analysis. The results of the experiment are given by the mean ± standard deviation. All data were tested for normality, and the homogeneity of the variance was tested prior to the analysis of variance. T test was used to compare the count data of two groups; the analysis of variance was used to compare the count data; and the Kaplan-Meier test was used to complete the survival analysis. The P value below 0.05 was considered to indicate a statistically significant difference. (* ($P < 0.05$), ** ($P < 0.01$), and *** ($P < 0.001$))

3. Result

3.1. High expression of ELOVL1 is correlated with the clinical pathological features of HCC patients

Analysis of related data from TCGA database by R software showed that ELOVL1 expressed differently in various cancer tissues and adjacent tissues (Fig. 1A), BLCA (BLCA, $p < 0.01$), breast cancer (BRCA, $p < 0.001$), bile duct cancer (CHOL, $p < 0.001$), and liver cancer (LIHC, $p < 0.001$). The relative expression of ELOVL1 in hepatic carcinoma tissues was significantly higher than that in normal liver and adjacent tissues (Fig. 1B, C, $p < 0.001$). GSE84402 data set in GEO database was selected to verify the expression of ELOVL1 in hepatocellular carcinoma. GSE84402 data set analyzed the differential expression of coding gene and noncoding RNA in hepatocellular carcinoma tissues and corresponding noncancerous tissues. After analysis, it was found that the results were consistent with TCGA database, and the expression of ELOVL1 in hepatocellular carcinoma was higher than that in normal tissues (Fig. 1D, p <

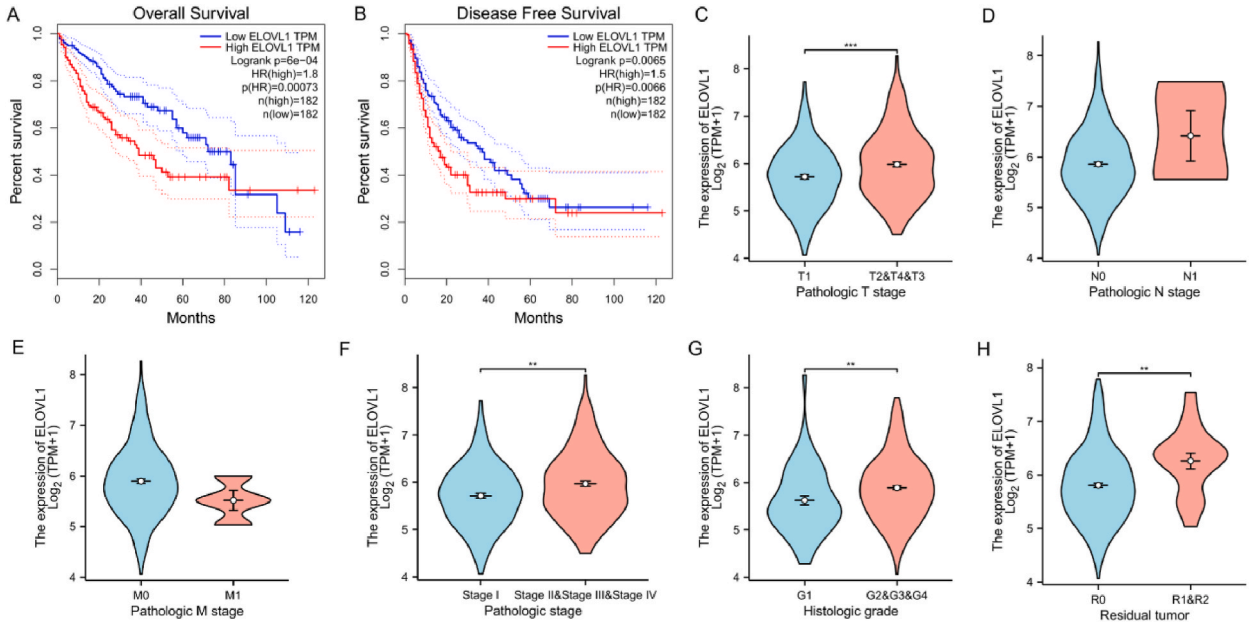

Fig. 2. GEPIA database analysis of the survival outcomes of hepatocellular carcinoma patients related to ELOVL1. (A) Overall survival rate of hepatocellular carcinoma patients with high expression of ELOVL1 compared to low expression patients. (B) Disease-free survival rate of hepatocellular carcinoma patients with high expression of ELOVL1 compared to low expression patients.(C) ELOVL1 and T stage of hepatocellular carcinoma.(D) ELOVL1 and N stageof hepatocellular carcinoma.(E) ELOVL1 and M stage of hepatocellular carcinoma.(F) ELOVL1 and pathologic stage of hepatocellular carcinoma.(G) ELOVL1 and histologic stage of hepatocellular carcinoma.(H) ELOVL1 and residual stage of hepatocellular carcinoma.

0.01) . We also drew a Receiver Operational Characteristic (ROC) curve to assess the diagnostic value of ELOVL1 by comparing ELOVL1 expression in adjacent HCC and normal liver tissue. The area under the curve (AUC) of ELOVL1 was 0.959 (CI = 0.931 to 0.986), suggesting high diagnostic potential (Fig. 1E).

3.2. High expression of ELOVL1 is associated with poor prognosis in HCC

Survival curves were plotted using the GEPIA database, which showed that the overall survival and disease-free survival were lower in patients with high levels of ELOVL1 than in those with low levels (Fig. 2A and B, $p < 0.01$). The clinical and gene expression data of 424 patients with liver cancer were collected from TCGA database. We found that the expression of ELOVL1 mRNA was significantly different among T1 and T2-T4 stages, I and II-IV stages of pathologic stage, G1 and G2-G4 stages of histologic stage and R0 and R1-R2 stages of residual stage(Fig. 2C–F-H,$p < 0.01$),however, there was no difference in expression between N stage and M stage(Fig. 2D and E). The association of ELOVL1 with the clinical characteristics was assessed by the Wilcoxon signed-rank test and Chi-Square test. As shown in Table 1, the results were significantly correlated with the pathologic stage ($p = 0.030$), body weight ($p < 0.001$), BMI ($p = 0.008$), histology type ($p = 0.037$), residual tumour ($p = 0.005$), pathologic grade ($p = 0.003$), blood vessel invasion ($p = 0.047$) and so on. Furthermore, as shown in Table 2, the tumor pathology stage ($p < 0.001$), the tumor condition ($p < 0.001$) and the expression of ELOVL1 ($p < 0.001$) were related to OS. Multivariate analysis indicated that tumor pathology stage ($p < 0.001$), tumor condition ($p = 0.003$) and ELOVL1 ($p < 0.001$) were independent prognostic factors for HCC.

3.3. ELOVL1 is upregulated in HCC tissues and HCC cell lines

The expression of ELOVL1 in hepatic carcinoma and normal tissues was examined in HPA database. The expression of ELOVL1 in

Table 1
The relationship between ELOVL1 mRNA expression and clinical characteristics in liver cancer.

Characteristics	Low expression of ELOVL1	High expression of ELOVL1	P value
n	187	187	
Pathologic stage, n (%)			0.030*
Stage I	99 (28.3 %)	74 (21.1 %)	
Stage II	39 (11.1 %)	48 (13.7 %)	
Stage III	35 (10 %)	50 (14.3 %)	
Stage IV	4 (1.1 %)	1 (0.3 %)	
Gender, n (%)			0.320
Female	56 (15 %)	65 (17.4 %)	
Male	131 (35 %)	122 (32.6 %)	
Age, n (%)			0.570
≤ 60	86 (23.1 %)	91 (24.4 %)	
>60	101 (27.1 %)	95 (25.5 %)	
Weight, n (%)			<0.001*
≤ 70	77 (22.3 %)	107 (30.9 %)	
>70	98 (28.3 %)	64 (18.5 %)	
BMI, n (%)			0.008*
≤ 25	76 (22.6 %)	101 (30 %)	
>25	92 (27.3 %)	68 (20.2 %)	
Histological type, n (%)			0.037*
Fibrolamellar carcinoma	3 (0.8 %)	0 (0 %)	
Hepatocellular carcinoma	183 (48.9 %)	181 (48.4 %)	
Hepatocholangiocarcinoma (mixed)	1 (0.3 %)	6 (1.6 %)	
Residual tumor, n (%)			0.005*
R0	172 (49.9 %)	155 (44.9 %)	
R1	3 (0.9 %)	14 (4.1 %)	
R2	1 (0.3 %)	0 (0 %)	
Histologic grade, n (%)			0.003*
G1	37 (10 %)	18 (4.9 %)	
G2	95 (25.7 %)	83 (22.5 %)	
G3	48 (13 %)	76 (20.6 %)	
G4	5 (1.4 %)	7 (1.9 %)	
AFP(ng/ml), n (%)			0.071
≤ 400	120 (42.9 %)	95 (33.9 %)	
>400	28 (10 %)	37 (13.2 %)	
Child-Pugh grade, n (%)			0.303
A	124 (51.5 %)	95 (39.4 %)	
B	9 (3.7 %)	12 (5 %)	
C	1 (0.4 %)	0 (0 %)	
Vascular invasion, n (%)			0.047*
No	117 (36.8 %)	91 (28.6 %)	
Yes	49 (15.4 %)	61 (19.2 %)	

hepatic carcinoma was higher than that in normal tissues (Fig. 3A and B). Immunohistochemical staining was performed on liver cancer tissues from both clinical and paired healthy tissues, and the results were in agreement with the HPA database (Fig. 3C and D). The expression of ELOVL1 mRNA and protein were determined by RT-PCR and Western blot, respectively. Both results indicated that ELOVL1 was more highly expressed in hepatic carcinoma cells than in normal hepatic cells (Fig. 3E, F, $p < 0.05$).

3.4. Transfection and screening of ELOVL1 interference lentivirus

In order to study the function of ELOVL1 in HCC, three recombinant interference lentiviruses carrying ELOVL1 (H_ELOVL1-shRNA507(PGMLV-ZsGreen-Puro), H_ELOVL1-shRNA680(PGMLV-ZsGreen-Puro), H_ELOVL1-shRNA567(PGMLV-ZsGreen-Puro)) and empty lentiviruses were transfected into Huh7 and HepG2 cells, respectively. After 72 h of transfection, puromycin was used to select successfully transfected cells, and then the fluorescent signals were observed under a fluorescence microscope to compare the transfection before and after, and observe the transfection of the cells (Fig. 4A and B). The downregulation of ELOVL1 mRNA and protein expression was confirmed by western blotting and RT-PCR, and H_ELOVL1-shRNA567 had the best interference efficiency (Fig. 4C–F, $p < 0.05$). Therefore, subsequent experiments were conducted using H_ELOVL1-shRNA567 lentivirus-transfected HCC cells.

3.5. ELOVL1 regulates the cell cycle process and apoptosis of HCC cells

Next, we will investigate the effects of ELOVL1 on HCC cell cycle and apoptosis. Flow cytometric analysis using PI assay indicated that the down-regulated expression of ELOVL1 inhibited G0/G1 cell cycle (Fig. 5A and B, $p < 0.05$). Using Annexin V/PI staining, the effect of ELOVL1 on cell apoptosis was determined by flow cytometry. The results indicated that the apoptosis rate of Huh7 and HepG2 cells after the transfection of ELOVL1 was higher than that in the control group (Fig. 5C, D, $P < 0.01$). In conclusion, ELOVL1 may regulate cell cycle and apoptosis in HCC.

3.6. ELOVL1 is involved in the growth and migration of HCC cells

The results of the scratch test indicated that ELOVL1 significantly reduced the capacity of cell repair and migration (Fig. 6A and B, $p < 0.01$). In the Transwell experiment, we found that the reduction of ELOVL1 significantly decreased the invasion capacity of ELOVL1 (Fig. 6C, D, $P < 0.001$). In the experiment, we found that the number of cells in the low expression group was smaller than that in the control group (Fig. 6E, F, $p < 0.01$). To sum up, our findings suggest that the reduced expression of ELOVL1 may inhibit Huh7 and HepG2 cells in vitro.

3.7. The relevant pathways of ELOVL1 based on KEGG and GSEA

Based on ELOVL1 and all its associated genes, we used R-language software to analyze KEGG and GSEA pathways to investigate the cellular mechanism of ELOVL1's role in liver cancer. As illustrated in Fig. 7A, ELOVL1 is associated with IL-17 signaling pathway, PPAR signaling pathway, and lipid metabolism. In addition, the expression of ELOVL1 may also influence the non-alcoholic fatty liver disease, the PI3K/AKT/mTOR signaling pathway, and the Wnt signaling pathway (Fig. 7B–F, $p < 0.01$). In the background, we have discovered that PI3K/AKT/mTOR signaling pathway is important in the development of cancer, NAFLD and fatty acid metabolism. The results showed that ELOVL1 was associated with PI3K/AKT/mTOR. In conclusion, it is suggested that ELOVL1 may play an important role in the development of liver cancer through regulation of PI3K/AKT/mTOR. Based on the mechanism of ELOVL1may be involved in this study, we investigate the effect of ELOVL1 on hepatic carcinoma via PI3K/AKT/mTOR signaling pathway.

3.8. ELOVL1 promotes tumor growth and metastasis by regulating the PI3K-AKT-mTOR signaling pathway

In order to study the molecular mechanism of ELOVL1 affecting the growth and invasion of HCC, we explored the relevant proteins of the PI3K/AKT/mTOR pathway. The PI3K/AKT/mTOR pathway is a prototype survival pathway involved in HCC carcinogenesis. We

Table 2
Correlations between overall survival and ELOVL1 mRNA expression using univariate and multivariate Cox regression.

Factor	Total (N)	Univariate analysis		Multivariate analysis	
		Hazard ratio (95 % CI)	P value	Hazard ratio (95 % CI)	P value
Age (≤ 60 vs > 60)	373	0.830 (0.585–1.176)	0.295		
Gender (Female vs Male)	373	0.793 (0.557–1.130)	0.200		
BMI (≤ 25 vs > 25)	336	0.798 (0.550–1.158)	0.235		
AFP(ng/ml) (≤ 400 vs > 400)	279	1.075 (0.658–1.759)	0.772		
Vascular invasion (No vs Yes)	317	1.344 (0.887–2.035)	0.163		
Pathologic stage (I& II vs III&IV)	349	2.504 (1.727–3.631)	<0.001*	2.161 (1.454–3.211)	<0.001*
Residual tumor (R0 vs R1&R2)	344	1.604 (0.812–3.169)	0.174		
Tumor status (Tumor free vs With tumor)	354	2.317 (1.590–3.376)	<0.001*	1.855 (1.242–2.771)	0.003*
ELOVL1 expression (high vs low)	373	2.055 (1.436–2.940)	<0.001*	2.055 (1.436–2.940)	<0.001*

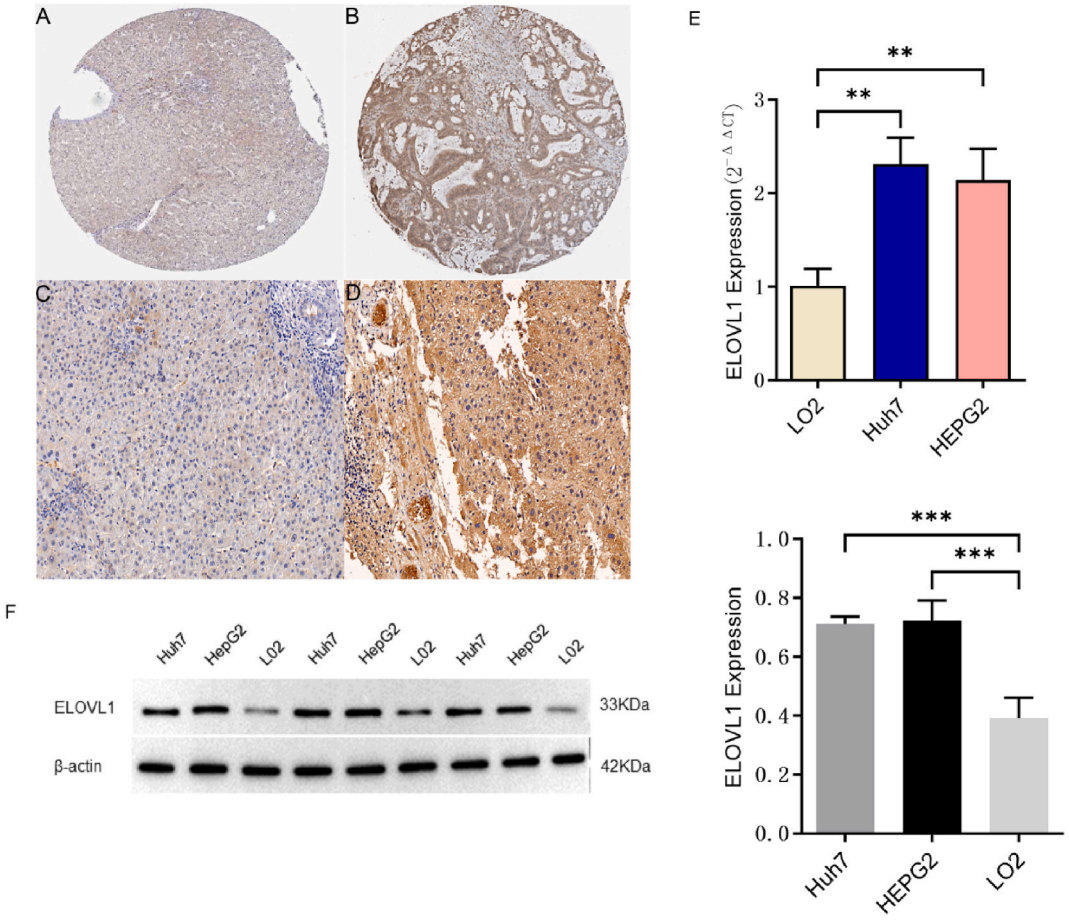

Fig. 3. Expression of ELOVL1 in hepatocellular carcinoma tissues and liver cancer cell lines
(A and B) ELOVL1 was detected by immunohistochemistry in HPA database; (C and D) ELOVL1 expression was confirmed by immunohistochemistry in clinical liver cancer and paired adjacent tissue; (E) ELOVL1 mRNA was detected by RT-PCR in normal hepatic (LO2) and hepatic carcinoma (Huh7, HepG2); (F) Western blot was used to detect the expression of ELOVL1 in normal hepatic (LO2) and hepatic carcinoma (Huh7, HepG2).

detected the phosphorylated protein level of PI3K/AKT/mTOR pathway in hepatocellular carcinoma tissues and adjacent tissues by immunohistochemical technique, and found that p-PI3K, *p*-AKT and *p*-mTOR were highly expressed in HCC tissues (Fig. 8 A)。 Western blot detection showed that the expression of phosphorylated PI3K (p-PI3K), AKT (*p*-AKT), and mTOR (*p*-mTOR) in the control group was higher than that in the lentivirus-transfected cell group (Fig. 8B–$p < 0.05$). In addition, we found that transfected cells showed lower activity of rapamycin, an mTOR inhibitor, at different concentrations, and exhibited higher cytotoxicity in the control group (Fig. 8C–$p < 0.05$). The above results indicate that the mechanism of action of ELOVL1 on liver cancer cells may be through the PI3K/AKT/mTOR pathway.

3.9. Effect of rapamycin on biological behavior of hepatocellular carcinoma cells

Through the above Western blot and cell activity experiments, we found that rapamycin can inhibit PI3K/AKT/mTOR pathway and affect the activity of hepatocellular carcinoma cells. Therefore, we will carry out wound healing and Transwell experiments on liver cancer cells treated with rapamycin. Results As shown in the following figure, rapamycin can reduce the invasive ability of hepatocellular carcinoma cells, while the invasive ability of cells in the low expression group of ELOVL1 treated with rapamycin is even lower (Fig. 9A,$p < 0.05$). The experimental results of wound healing show that rapamycin can reduce the migration ability of hepatocellular carcinoma cells, and the migration ability of cells in the low expression group of ELOVL1 treated with rapamycin is even lower(Fig. 9B, $p < 0.05$). Through the above experiments, we believe that ELOVL1 can regulate PI3K/AKT/mTOR signaling pathway, and can reduce the malignant biological behavior of hepatocellular carcinoma cells together with rapamycin.

3.10. ELOVL1 can inhibit the proliferation of liver cancer cells in the body

After inoculation of liver cancer cells from blank group, control group, and experiment group, we observed the growth of the tumor

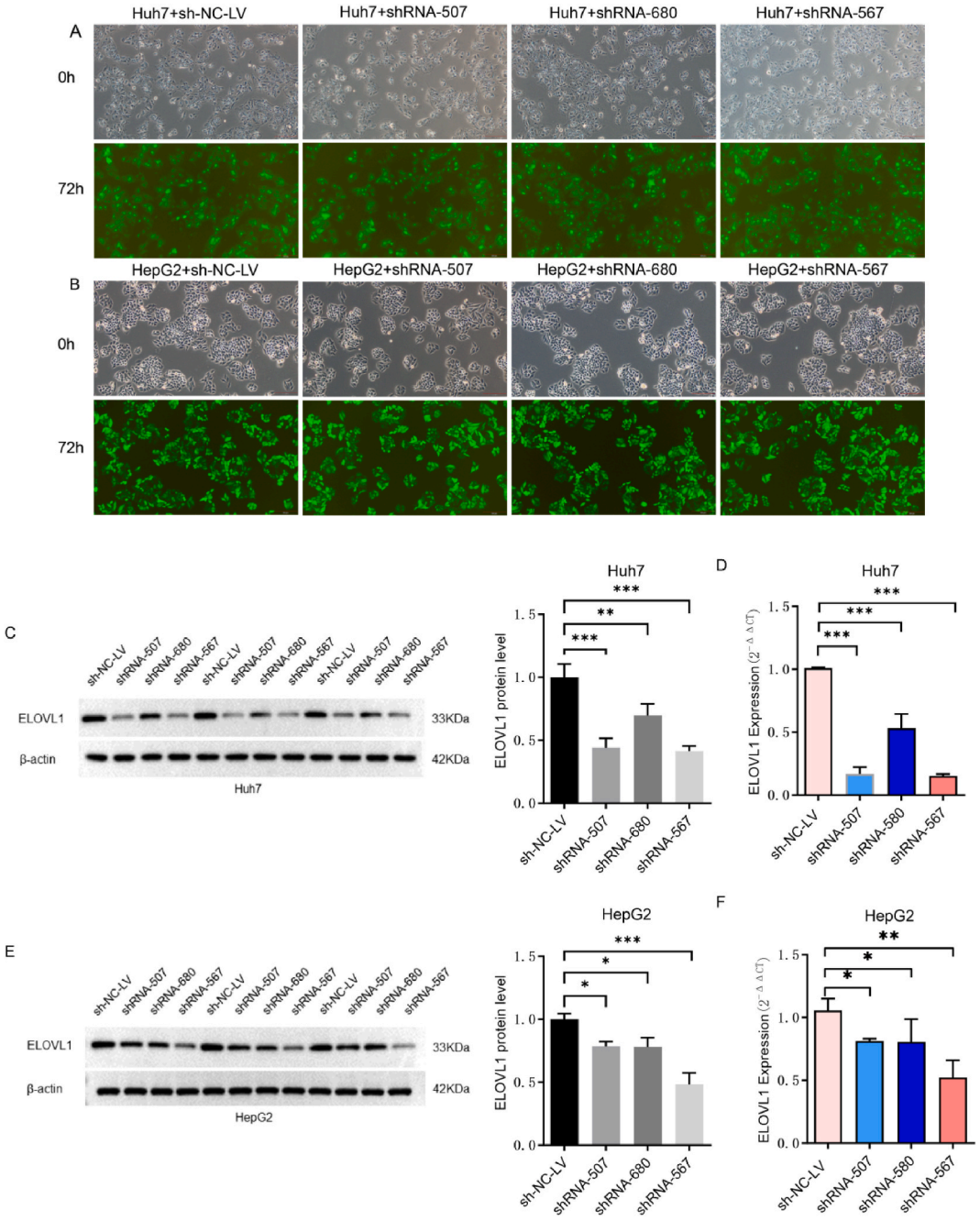

Fig. 4. Transfection and screening of interfering lentivirus.
(A and B) Using fluorescence microscopy to observe the transfection of empty and interfering lentiviruses in HCC cells; (C and E) Western blotting to detect the protein expression of ELOVL1 in HCC cells after lentivirus transfection; (D and F) RT-PCR to detect the mRNA expression of ELOVL1 in HCC cells after lentivirus transfection.

in each group. Tumor volume was measured every 3 days, and the growth curve was plotted. After 21 days of culture, the nude mice were euthanized with cervical dislocation, and the subcutaneous tumors were dissected, weighed and photographed (Fig. 10A and B.). The results showed that the growth rate of subcutaneous tumors in the low expression group of ELOVL1 was lower than that in the control group, the size of the tumor was smaller, and the tumor weight was lower (Fig. 10C, D, $p < 0.05$). Immunohistochemical method was used to detect the expression of ELOVL1, p-PI3K, *p*-AKT, and *p*-mTOR. The results showed that the lentivirus expression was lower in the experimental group than in the control group (Fig. 10 E).

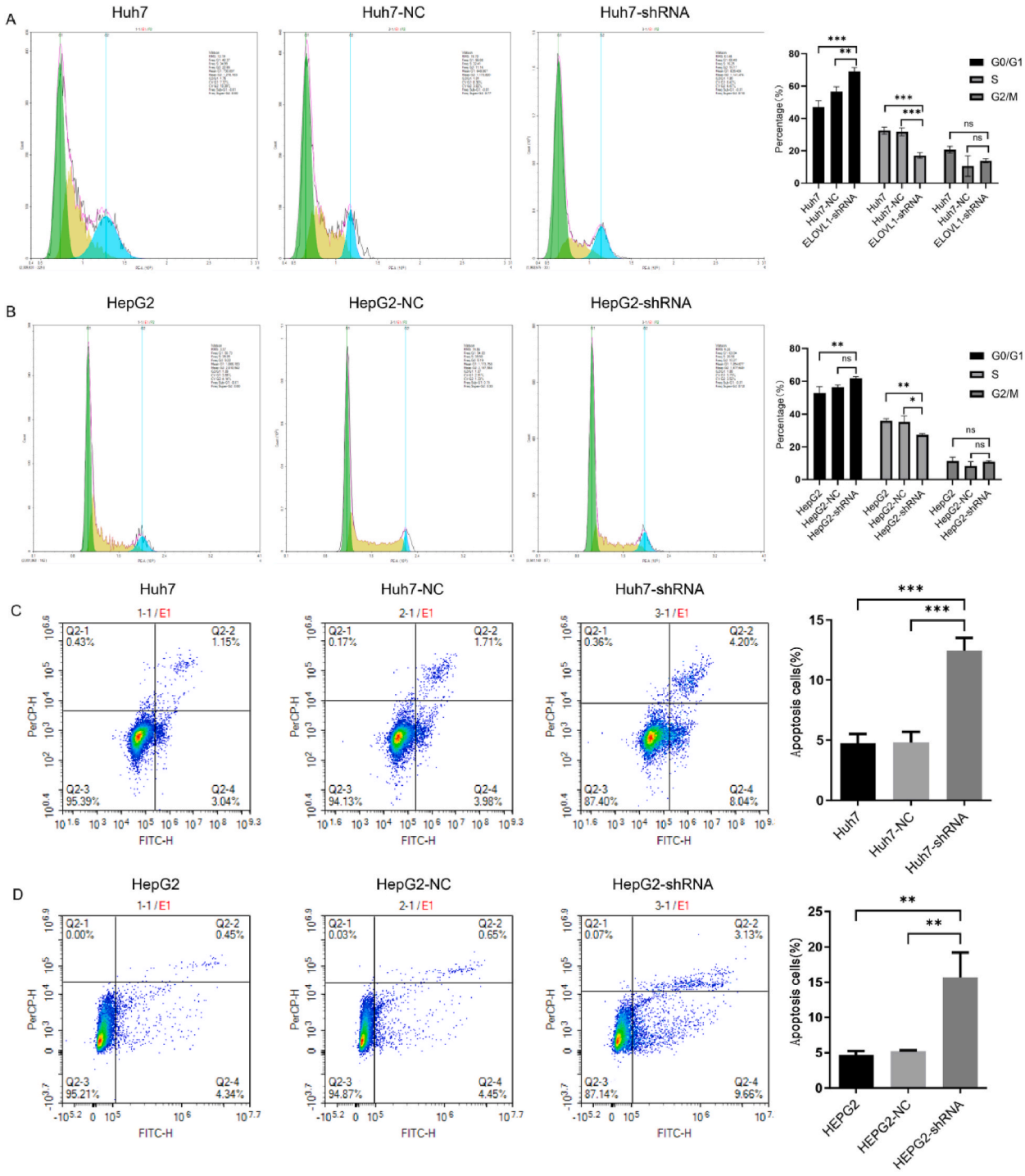

Fig. 5. ELOVL1 expression affects the cell cycle and apoptosis of HCC cells.
(A and B) The silence of ELOVL1 on the cell cycle of HCC; (C and D) Cell apoptosis experiment validates the effect of high or low expression of ELOVL1 on HCC cells.

4. Discussion

The onset of cancer is intricately linked to substantial modifications in cellular metabolism, encompassing variations in lipid, carbohydrate, nucleic acid, and amino acid metabolism [30]. Lipids are a diverse group of molecules that primarily include fatty acids (FAs), cholesterol, and phospholipids (PLs). They are important in many physiological processes, including energy storage and

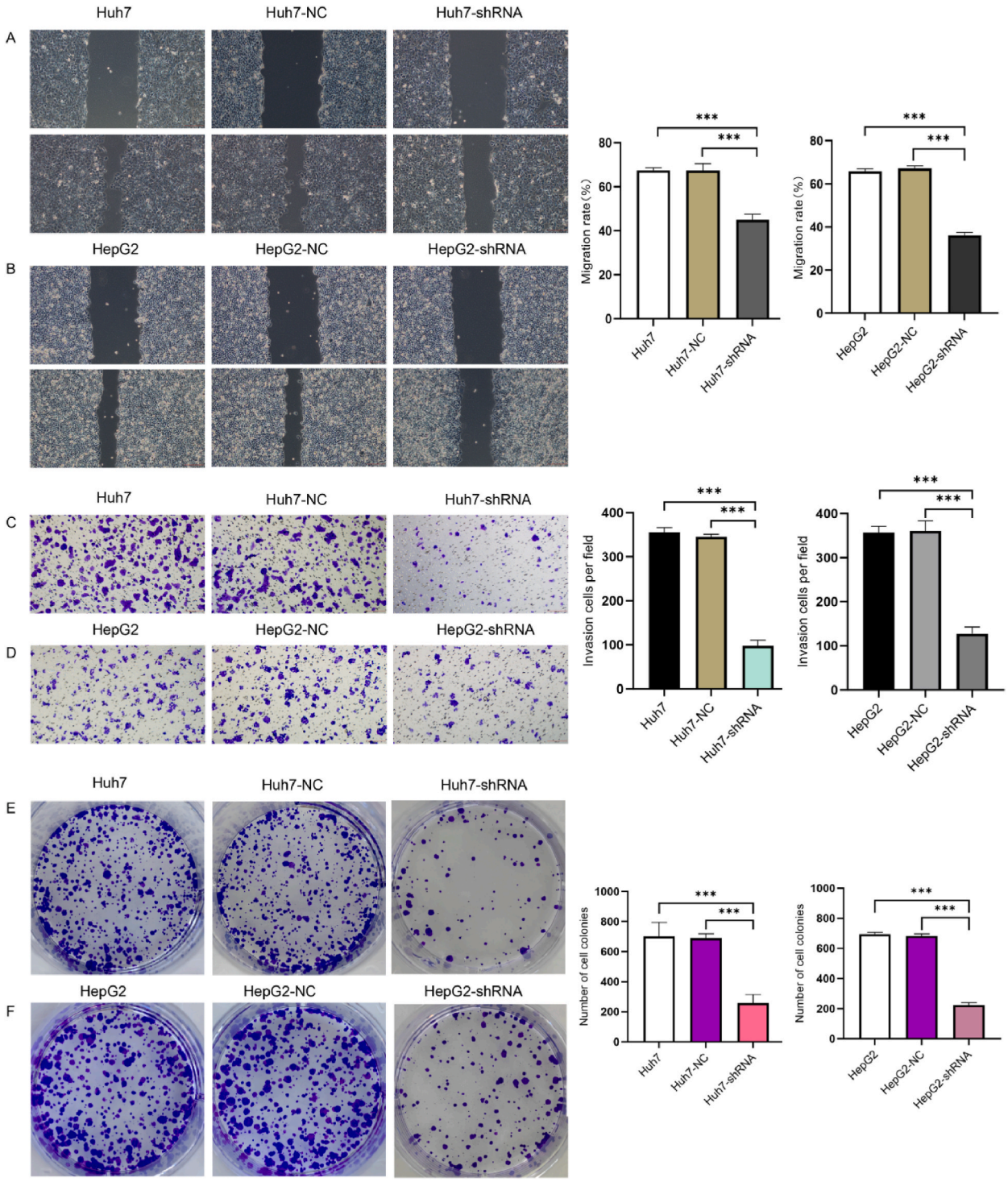

Fig. 6. The expression of ELOVL1 influences the biological behavior of HCC cells.
(A and B) Scratch test was used to detect the migration ability of HCC cells after silencing ELOVL1; (C and D) After silencing ELOVL1, the migration and invasion abilities of HCC cells were detected through Transwell; (E and F) Cell clone experiment verified the changes in the proliferation and growth ability of HCC cells after silencing ELOVL1.

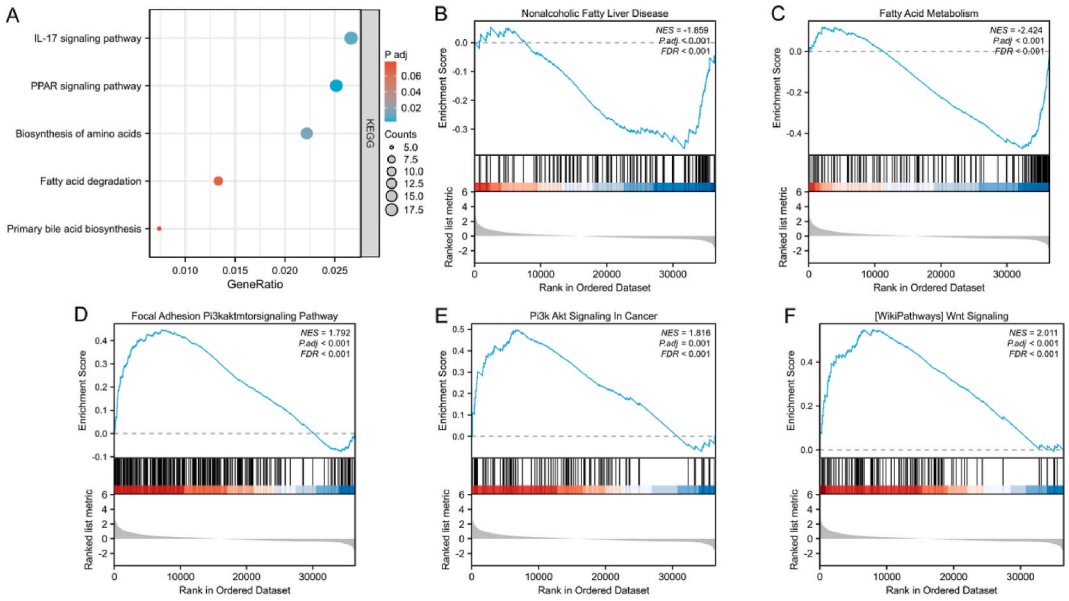

Fig. 7. Enrichment Analysis of ELOVL1 Function in Hepatocellular Carcinoma.
(A) KEGG enrichment analysis; (B) Nonalcoholic fatty liver disease; (C) Fatty acid metabolism; (D) Focal adhesion PI3K-AKT-mTOR signaling pathway; (E) PI3K-AKT signaling in cancer; (F) Wnt signaling.

metabolism, epigenetic regulation, signal transduction, immune modulation, inflammatory reaction, and cell recognition. Through these physiological processes, lipids are essential in the development, progression, and maintenance of cancer [31,32]. The ELOVL family of elongases is a major rate-limiting enzyme in the biosynthesis of very long-chain fatty acids, which regulates the rate and direction of this metabolism. The ELOVL family is involved in a variety of diseases and cancers. The role of ELOVL2 in regulating tumor suppressor INPP4B in prostate cancer progression [33]. NOTCH-RIPK4-IRF6-ELOVL4 gene blockade impedes cancer progression [34]. Not only was ELOVL1 highly expressed and promoted in colorectal and breast cancer, but it was also found that the mRNA expression of HCC was higher than that of adjacent normal hepatic tissue. However, the clinical significance of ELOVL1 and its biological role in HCC are still unknown.

The study revealed that among 113 cases of HCC analyzed using immunohistochemical chips, ELOVL1 exhibited high expression in 68 cases and low expression in 45 cases [19]. We also found that the expression of ELOVL1 in HCC was significantly elevated by immunohistochemical method. In addition, RT-PCR and Western blot were used to confirm that the expression of ELOVL1 was up-regulated in Huh7 and HepG2 hepatic HCC in comparison with LO2. In summary, our findings indicate frequent over-expression of ELOVL1 in HCC tissues and cells.

Analysis of the data from an online database revealed that the expression of ELOVL1 was associated with clinical pathology and prognosis. There was significant correlation between the expression of ELOVL1 and pathologic stage, body weight, BMI, histology type, residual tumor, histologic grade and vascular invasion. Furthermore, we analyzed the difference of ELOVL1 expression and clinical variable grouping. The results showed that ELOVL1 was more highly expressed in T, pathologic, histologic and residual tumor. The results showed that the expression of ELOVL1 could not only influence the clinical features of patients, but also the stage of cancer. Therefore, the high expression of ELOVL1 might promote the progression of HCC and influence the prognosis of HCC. In addition, the multi-factorial Cox analysis confirmed that ELOVL1 was a separate risk factor for poor overall survival in HCC. Our results indicate that ELOVL1 may contribute to HCC progression. In this study, we found that silencing ELOVL1 expression in Huh7 and HepG2 cells could not only inhibit the proliferation, apoptosis and cell cycle, but also inhibit metastasis and invasion. Moreover, the low expression of ELOVL1 also inhibited the growth of xenograft tumors in vivo. Our findings indicate that ELOVL1 might be a potential tumor promoter for the development of liver cancer. However, it is not yet known how ELOVL1 inhibits HCC cell growth and migration.

The PI3K/AKT/mTOR signaling pathway is commonly dysregulated in various human tumors, thus, its dysregulation is widely recognized as a crucial mechanism in tumorigenesis. Changes in the function of specific genes in the PI3K/AKT pathway can result in cellular transformation, which regulates cancer cell apoptosis and proliferation. Additionally, this pathway is closely linked to tumor invasion and metastasis [32,35]. Mammalian target of rapamycin (mTOR), a component of this complex, plays a central role in regulating cell survival and metabolic processes by integrating inputs from amino acids, nutrients, and extracellular signals [36]. It promotes tumor development by regulating the AGC family of serine/threonine kinases, with a particular emphasis on the AKT protein. Additionally, research has demonstrated that mTOR facilitates the formation of lipids essential for growth and energy, further promoting cancer [37,38]. ELOVL1 regulates lipid metabolism and may promote cancer by affecting mTOR through this pathway. Through bioinformatics analysis, we hypothesize that ELOVL1 may modulate HCC cells via the PI3K/AKT/mTOR signaling pathway. We detected the expression of phosphorylated PI3K, AKT and mTOR in hepatocellular carcinoma, and found that the expression was

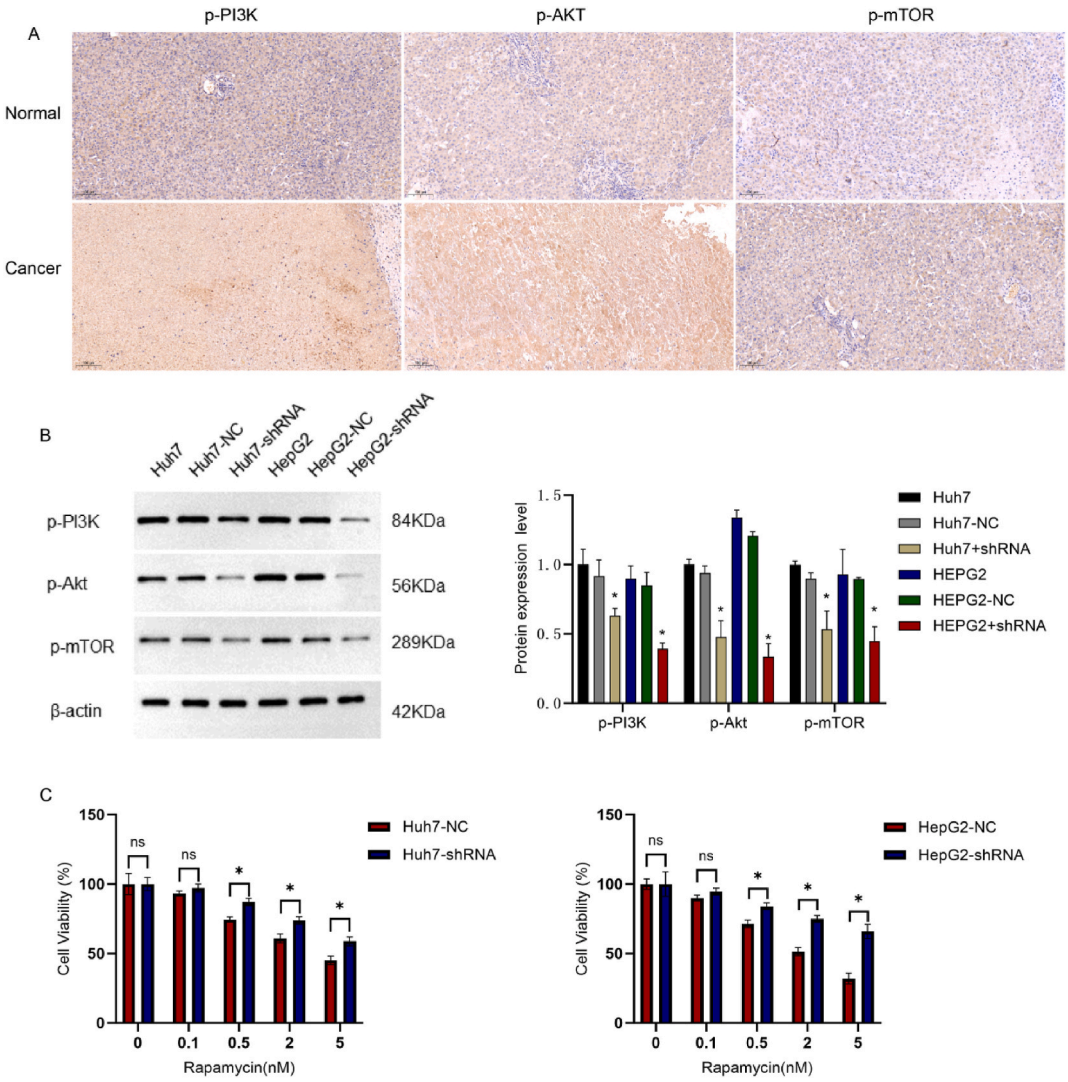

Fig. 8. ELOVL1 inhibits tumor growth and metastasis by regulating the PI3K-AKT-mTOR signaling pathway. (A) Expression of phosphorylated protein of PI3K/AKT/mTOR pathway in hepatocellular carcinoma (B) After silencing ELOVL1, the related proteins of the PI3K/AKT/mTOR pathway were detected by Western blotting; (C) The cytotoxic effect of the mTOR inhibitor rapamycin at different concentrations on ELOVL1-silenced HCC cells was detected by CCK8 experiment.

up-regulated. The silencing of ELOVL1 led to a reduction in the expression and activity of p-PI3K, *p*-AKT, and *p*-mTOR, ultimately resulting in the inactivation of the pathway. By detecting the phosphorylated protein level of this pathway in allograft, it was found that the expression in ELOVL1 silent tissue decreased, which was consistent with the experimental results in vitro. Additionally, when treating liver cancer cell lines with the mTOR inhibitor rapamycin, the results showed that ELOVL1-silenced liver cancer cells had reduced sensitivity to rapamycin compared to the control group. In the subsequent wound healing experiment and transwell experiment, it was found that rapamycin could reduce the invasion and migration ability of hepatocellular carcinoma cells, especially in ELOVL1 silent cells. Thus, the up-regulation of ELOVL1 expression can activate PI3K/AKT/mTOR signaling pathway in hepatocellular carcinoma and enhance the cytotoxicity of rapamycin on HCC cells.

It is found that the high expression of ELOVL1 may regulate PI3K/AKT/mTOR signaling pathway to promote the occurrence and development of hepatocellular carcinoma. In addition, the expression level of ELOVL1 is related to the prognosis of patients with hepatocellular carcinoma, so ELOVL1 may become a new target for the diagnosis or treatment of hepatocellular carcinoma. However, there are still many shortcomings in this study. For example, the clinical data related to hepatocellular carcinoma are all obtained by public database analysis. Whether the patients with hepatocellular carcinoma in our hospital meet the clinical characteristics needs to collect more clinical data of patients and analyze and verify the results of return visits. In the experiment, we only verified the regulation of ELOVL1 on PI3K/AKT/mTOR pathway in hepatocellular carcinoma cell lines, and did not further analyze and verify the related genes or pathways upstream and downstream of ELOVL1. Our research is limited to the level of cells and animals, and it is

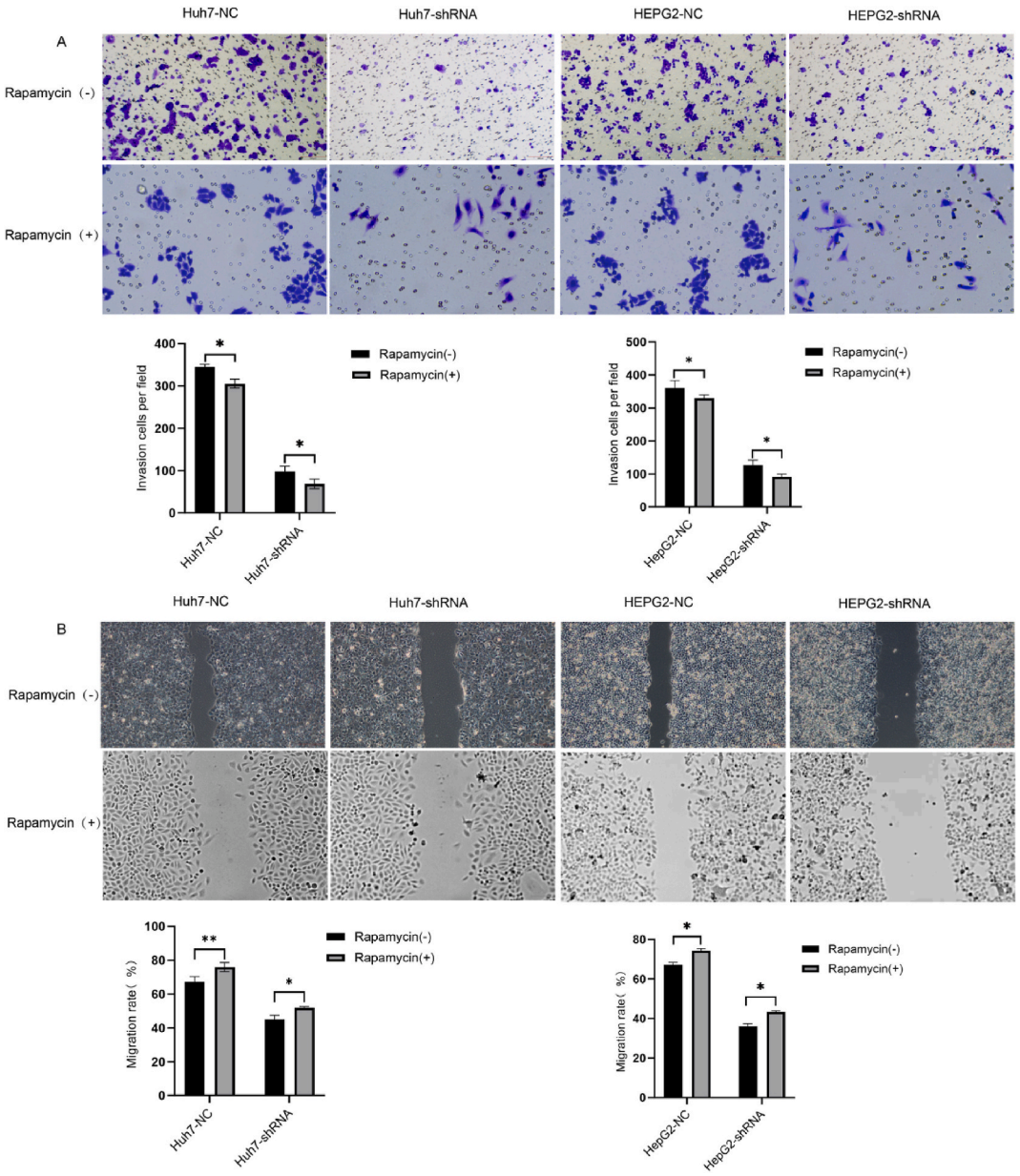

Fig. 9. Effect of rapamycin on biological behavior of hepatocellular carcinoma cells. (A) Effect of rapamycin on invasion ability of hepatocellular carcinoma cells; (B) Effect of rapamycin on migration ability of hepatocellular carcinoma cells.

necessary to further explore the role of ELOVL1 and its metabolite ultra-long chain fatty acids in patients with hepatocellular carcinoma, such as whether the concentration of ultra-long chain fatty acids in blood can be used as a method for predicting or diagnosing hepatocellular carcinoma; Whether reducing the concentration of ultra-long chain fatty acids can weaken the progress of hepatocellular carcinoma. In order to determine the functional mechanism of ELOVL1 in the occurrence and development of hepatocellular carcinoma, and to find new diagnosis or treatment methods for patients with hepatocellular carcinoma.

5. Conclusion

In conclusion, the present study provides the first evidence that ELOVL1 regulates the up-regulation of PI3K-AKT-mTOR cell carcinoma and promotes the growth of cancer.

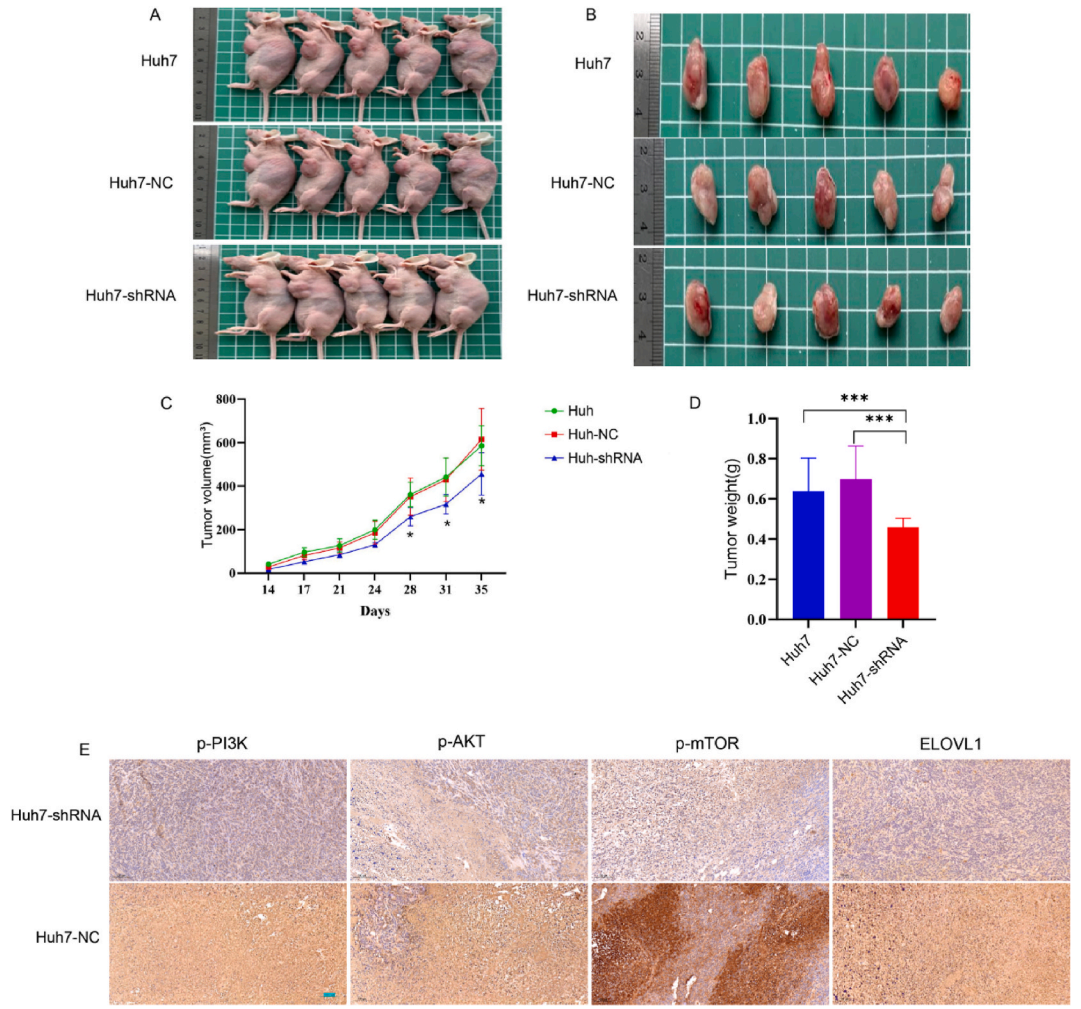

Fig. 10. Low expression of ELOVL1 inhibits tumor growth in vivo. (A) Tumor-bearing nude mice euthanized by cervical dislocation method; (B) Dissected tumor tissue; (C and D) Comparison of tumor growth curve and tumor weight; (E) Expression of ELOVL1, p-PI3K, *p*-AKT and *p*-mTOR in transplanted tumor.

Ethics approval and consent to participate

The study was approved by the Ethics Committee of Affiliated Hospital of Guizhou Medical University, and all participants signed an informed consent form. All animal experimental schemes have been approved by the Animal Ethics Committee of Guizhou Medical University.

Consent for publication

All participants signed an informed consent form, and consented to publication.

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Ethics approval

This study was approved by the Ethics Committee of the Affiliated Hospital of Guizhou Medical University (Approval Number: 2023-628).

This study was approved by the Animal Ethics Committee of Guizhou Medical University (approval number: 2304736).

CRediT authorship contribution statement

Liang Qin: Writing – review & editing, Visualization, Validation, Methodology. **Cheng-ze Song:** Validation, Methodology. **Fa-yang Yuan:** Visualization, Validation. **Xue-fa Wang:** Validation. **Yang Yang:** Validation. **Yi-fei Ma:** Writing – original draft. **Zi-li Chen:** Writing – original draft.

Declaration of competing interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests:Zi-Li Chen reports financial support was provided by Guizhou Medcial University. If there are other authors, they declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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N/A.

Abbreviations

ELOVL1	Elongase of Verylong Chain Fatty Acids 1
DSS	Disease Free Survival
GEPIA	Gene Expression Profiling Interactive Analysis
GSEA	Gene Set Enrichment Analysis
HPA	Human Protein Atlas
OS	Overall survival
HCC	Hepatocellular Carcinoma

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