Dysregulation of the SREBP pathway is associated with poor prognosis and serves as a potential biomarker for the diagnosis of hepatocellular carcinoma

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Abstract. Hepatocellular carcinoma (HCC) is a severe disease associated with a poor prognosis. The role of aberrant lipid metabolism in the development and progression of HCC necessitates detailed characterization. Sterol regulatory element-binding proteins (SREBPs), pivotal transcription factors governing lipogenesis, are central to this process. The present study aimed to assess the regulation of HCC by the SREBP signaling pathway, examining the expression levels of genes in this pathway, the clinical implications and its prognostic value using the Kaplan-Meier method. Pearson's correlation coefficient was used to identify the co-expression of SREBP pathway genes in HCC. Genomic analysis examined the frequency of TP53 mutations in groups with and without SREBP pathway alterations. In addition, small interfering RNAs targeting genes of the SREBP pathway were transfected into Huh-7 and HCC-LM3 cell lines. Subsequently, Cell Counting Kit-8 and Transwell assays were carried out to evaluate the viability and invasion of these cells. Reverse transcription-quantitative PCR and western blotting were performed to investigate the expression of TP53 in response to silencing of SREBP pathway genes. Dysregulation of SREBP pathway genes was detected in HCC tissues compared with in normal liver tissues, and predicted a poor prognosis. Silencing these genes reduced the viability and invasion of HCC cells. Furthermore, abnormal SREBP pathway gene expression was associated with poor survival rates, vascular invasion, advanced tumor stage and an increased incidence of TP53 mutations. By contrast, knockdown of SREBP pathway genes decreased mutant TP53 expression at both the mRNA and protein levels in HCC cells. The findings of the present study suggested that SREBP pathway genes could serve as promising prognostic biomarkers for HCC. The combined analysis of individual gene expression levels offers offer novel insights into the pathogenesis and progression of HCC.

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Introduction

Liver cancer is a global disease and reports have estimated that >1 million individuals will be affected by liver cancer annually by 2025 (1,2). Hepatocellular carcinoma (HCC) is the most common form of liver cancer, accounting for ~90% of cases (3). Excessive alcohol consumption, obesity, and hepatitis B or C infection are risk factors for HCC (3). Combined therapy with sterol regulatory element-binding protein (SREBP) inhibitors and the use of new serum biomarkers in early diagnosis has led to an improvement in the survival of patients with HCC. However, the side effects, such as altered lipid metabolism and gastrointestinal issues, and unfavorable prognosis indicate the importance of understanding the molecular pathogenesis of HCC, which may further link molecular subtypes with specific therapies. Studies have revealed that abnormal gene expression of fatty acid metabolism can trigger the progression of HCC (4,5). Hence, investigating the dysregulated genes during HCC development may help advance diagnosis and translate into clinical treatment to improve patient outcomes.

Increasing evidence has suggested that dysregulation of lipid metabolism leads to tumor development (6,7). Unlike normal cells, HCC cells exhibit an elevated *de novo* lipid synthesis rate, causing fatty acid upregulation and accumulation *in vivo* (4,8). Several transcription factors are key in regulating lipid metabolism, and among them, SREBPs are some of the key factors in lipid synthesis and signaling transduction (9). There are three subtypes in the SREBP family, SREBP-1a and SREBP-1c, which result from alternative splicing of SREBPF1 transcripts, and SREBP2, which

is the translated protein of the SREBPF2 gene (10,11). Upon the elevation of sterol, SREBP cleavage-activating protein (SCAP) binds to SREBP and insulin-induced genes (INSIG; encoded by INSIG1 and INSIG2), to facilitate escorting of the complex from the endoplasmic reticulum (ER) membrane to the Golgi apparatus (12,13). If sterol levels are depleted, membrane-bound ubiquitin ligase autocrine motility factor receptor (AMFR) competes with SCAP to bind INSIG, consequently blocking SREBP translocation (14). The SREBP pathway is regulated through several mechanisms, including the PI3K/AKT pathway, insulin-related mTORC1 regulation and microRNA-mediated regulation (15,16). The SREBP pathway can protect tumor cells from nutritional shortages by supplying energy, lipids and other necessary factors.

Given the role of the SREBP pathway in tumors, drugs targeting SREBPs have been developed; however, direct targeting of the SREBP transcription factors is difficult (17). Current strategies focus on inhibiting the transport of SREBPs or cleavage enzymes to block activation. Notably, the action of only a few SREBP inhibitors have been studied in tumors, for example, betulin has been reported to inhibit HCC progression by suppressing the SREBP pathway in mice (18,19). Therefore, understanding the upstream and downstream pathways that regulate the SREBP pathway warrant further investigation. A previous study reported that phosphoenolpyruvate carboxykinase 1 (PCK1) is phosphorylated by activated AKT, then translocates to the ER to phosphorylate INSIG1, followed by activation of SREBP proteins (20). In addition, another factor, SREBP regulating gene (SPRING), has been identified as a previously unrecognized factor that governs SREBP activity *in vivo* in mice by controlling the levels of functional SCAP (21). These findings indicate that both the classic SREBP pathway and its recently identified regulators serve a crucial role together in HCC. However, the clinical application of the SREBP signaling pathway in patients with HCC remains unknown.

In the present study, bioinformatics analysis of a large number of samples was used to evaluate the clinical importance of the SREBP pathway in HCC. Furthermore, the potential targets and underlying mechanisms of the pathway dysregulation in HCC were also investigated.

Materials and methods

Acquiring data from The Cancer Genome Atlas (TCGA), Gene Expression Omnibus (GEO) and Human Protein Altas (HPA). RNA-seq data [gene expression: Fragments per kilobase of exon per million mapped reads (FPKM)] and clinical information of 424 liver samples (50 healthy control tissues, 371 primary tumors and 3 recurrent tumors) were downloaded from TGCA database (project TCGA-LIHC; https://portal.gdc.cancer.gov/). The 3 recurrent tumor samples were excluded from downstream analysis, which left 371 valid tumor samples. Expression of SREBP pathway-related genes was filtered from the dataset, and significance between the normal samples and tumor samples was assessed by unpaired Student's t-test in R. (version 3.4.2; www.r-project.org) Plots were generated using the ggpubr (https://rpkgs.datanovia.com/ggpubr/) and ggplot2 packages in R and receiver operating characteristic (ROC) curves were calculated and plotted using the pROC package in R (22). Expression levels, grouped as high and low, of genes were assigned by the optimal threshold determined in X-tile software (ver. 3.6.1) (23). Survival rate plots were generated using the autoplot function of the R packages ggplot2 (https://ggplot2.tidyverse.org/) and ggfortify (https://cran.r-project.org/web/packages/ggfortify/index.html). A dataset using SCAP-knockout mice in the GEO database (GSE169104; https://www.ncbi.nlm.nih.gov/geo/) was identified and analyzed using RNA-seq analysis (24). Specifically, raw RNA-seq reads were cleaned using fastp (v0.20.0) (25) program, and aligned to mm10 using STAR (v2.7.3a) (26). Only unique mapped reads were kept, and FPKM were calculated by stringtie (v2.0) (27-29). Additionally, protein expression of the SREBP pathway was determined by acquiring immunohistochemistry of normal and tumor tissues from the HPA database(https://www.proteinatlas.org/).

Survival analysis. A survival probability plot was plotted against alcohol consumption and tumor stages using the web tool Kaplan-Meier plotter (http://kmplot.com/analysis) and log-rank test was utilized to obtain P-values (30-32). The RNA-seq datasets of 364 patients with available clinical data in this web tool database were used for this analysis. For the generation of Kaplan-Meier plots, patient data were split by cut-off values between upper and lower quartiles, and the best threshold was used.

Analysis of mutations and dysfunction of SREBP pathway genes in HCC. mRNA data (gene expression: FPKM) and clinical information of 1,043 samples from four databases [Liver Hepatocellular Carcinoma (TCGA, Firehose Legacy; https://gdac.broadinstitute.org/runs/stddata__2016_01_28/data/LIHC/20160128/); and three other studies (33-35)] were analyzed. A total of 8 genes including core SREBP pathway signaling and newly identified regulators were chosen to query in these databases. All the combined study analyses excluded overlapping samples and patients. Samples with at least one alteration in the queried genes were grouped as the 'altered group', and samples with no alterations in the queried genes were grouped as the 'unaltered group'. The survival, genomic alteration, co-expression, enrichment of dysregulated genes in different groups and mutations were analyzed with the cBioPortal for Cancer Genomics web-based tool (https://www.cbioportal.org).

Cell culture and transfection. The human HCC cell lines Huh-7 and HCC-LM3, which were obtained from The Cell Bank of Type Culture Collection of The Chinese Academy of Sciences, were cultured in Dulbecco's modified Eagle's medium (DMEM; Sigma-Aldrich; Merck KGaA) supplemented with 10% fetal bovine serum (BIOEXPLORER Life Sciences) and 1% penicillin-streptomycin (Gibco; Thermo Fisher Scientific, Inc.). Huh-7 and HCC-LM3 cells were cultured in a humidified incubator (Thermo Fisher Scientific, Inc.) containing 5% CO_2 at 37˚C. To silence SREBP1, SREBP2, SCAP, AMFR, SPRING and INSIG2, two small interfering RNAs (siRNAs) for each gene were designed by Guangzhou RiboBio Co., Ltd., and the two siRNAs were combined to form the third siRNA for each gene. Target sequences of the siRNAs are listed in Table SI. The siNC (cat. no. siN0000001-1-5) was

also provided by Guangzhou RiboBio Co., Ltd. Cell transfection was carried out using Lipofectamine® 3000 (Invitrogen; Thermo Fisher Scientific, Inc.), according to the manufacturer's instructions. Briefly, $2.5x10^5$ cells were seeded in 6-well plates and were transfected with 7.5 pmol siRNA, in an atmosphere containing 5% CO_2, at 37˚C. Reverse transcription-quantitative PCR (RT-qPCR), Cell Counting Kit-8 (CCK-8) and Transwell invasion assays were performed 24 h after transfection, and western blotting was performed 48 h after transfection. The knockdown efficacy of specific siRNAs was validated by RT-qPCR and western blotting. The most effective siRNA out of the three for each gene was used in the Transwell invasion assay and in western blotting.

CCK-8 assay. Transfected Huh-7 and HCC-LM3 cells were cultured for 24 h and were then seeded in 96-well plates ($1x10^4$ cells/well). After growing for 24 h, the cells were incubated with CCK-8 solution (Shanghai Yeasen Biotechnology Co., Ltd.) at a volume of 10 μl/well for 2 h. Absorbance at a wavelength of 450 nm was detected for cell viability analysis.

Transwell invasion assay. According to RT-qPCR validation, the most effective siRNA for each gene was used to transfect the Huh-7 and HCC-LM3 cells. For the invasion assay, a 1:9 dilution of Matrigel (BD BioCoat; Corning, Inc.) was prepared in serum-free DMEM, and 100 μl of the diluent was used to coat the bottom of the Transwell chamber (Guangzhou Jet Bio-Filtration Co., Ltd.) for 1 h at 37˚C. Subsequently, $5x10^4$ transfected Huh-7 and HCC-LM3 cells were seeded in the upper chamber and DMEM supplemented with 10% fetal bovine serum was added to the lower chamber, and the cells were incubated at 37˚C in a 5% CO_2 incubator. After 48 h, the cells were fixed with 4% paraformaldehyde for 20 min and stained with crystal violet for 15 min at room temperature. After fixation and staining, three fields were randomly selected under a light microscope and the number of cells penetrating the filter membrane was counted; the fields were analyzed using ImageJ 2.9.0 software (National Institutes of Health).

RT-qPCR analysis. Huh-7 and HCC-LM3 cells were transfected for 24 h, after which, total RNA was extracted from the cells using TRIzol® (Invitrogen; Thermo Fisher Scientific, Inc.) according to the manufacturer's protocol. Subsequently, total RNA was reverse transcribed to cDNA using HiScript II Q RT SuperMix (Vazyme Biotech Co., Ltd.) according to the manufacturer's protocol. qPCR was performed at 95˚C for 180 sec for initial denaturation, followed by 40 cycles of denaturation at 95˚C for 15 sec and annealing and extension at 60˚C for 30 sec. SYBR Green-based RT-qPCR (Selleck Chemicals) was conducted to examine the relative mRNA expression levels of SREBP1, SREBP2, SCAP, AMFR, SPRING, INSIG2 and TP53, which were normalized to β-actin. The data were calculated and analyzed using the $2^{-\Delta\Delta Cq}$ method (36). The primers used are listed in Table SII.

Western blotting. Huh-7 and HCC-LM3 cells were transfected for 48 h, then, the cells were lysed using RIPA buffer (Beyotime Institute of Biotechnology) containing protease inhibitors, and proteins were obtained by centrifugation at 14,000 x g for 15 min at 4˚C. Total protein was quantified with a BCA assay kit (Beyotime Institute of Biotechnology). The proteins lysates (20 μg) were then separated by SDS-PAGE (Omni-Easy™; Shanghai Epizyme Biomedical Technology Co., Ltd.) on 10% gels and transferred onto PVDF membranes (Merck KGaA). The PVDF membranes were blocked in Tris-buffered saline containing 1% Tween-20 (TBST, pH 7.4) with 5% non-fat milk for 1 h at room temperature and incubated with primary antibodies overnight at 4˚C. The primary antibodies used in the present study were: SREBP-1 (dilution 1:200; cat. no. sc-365513; Santa Cruz Biotechnology, Inc.), SREBP-2 (dilution 1:1,000; cat. no. MABS1988; Sigma-Aldrich; Merck KGaA), AMFR (dilution 1:1,000; cat. no. 16675-1-AP; Proteintech Group, Inc.), SPRING (dilution 1:1,000; cat. no. orb1165; Biorbyt, Ltd.), TP53 (dilution 1:10,000; cat. no. 60283-2-Ig; Proteintech Group, Inc.) and β-actin (dilution 1:5,000; cat. no. A5441; Sigma-Aldrich; Merck KGaA). After washing with TBST three times, the membranes were incubated for 1 h at room temperature with the following secondary antibodies: HRP-conjugated Goat Anti-Mouse IgG (dilution 1:10,000; cat. no. SA00001-1; Proteintech Group, Inc.) or HRP-conjugated Goat Anti-Rabbit IgG (dilution 1:10,000; cat. no. SA00001-2; Proteintech Group, Inc.). Protein band detection was carried out using a Gel Imaging System (Tanon Science and Technology Co., Ltd), and the bands were analyzed by densitometry using ImageJ software (National Institutes of Health). β-actin was used as an internal control.

Bioinformatics analysis. The ROC curve was used to determine the diagnostic value of genes in HCC, and the area under the curve (AUC) was calculated. The relationships between the mRNA expression levels of different SREBP pathway genes in HCC were assessed through Pearson correlation analysis. SPSS 22.0 (IBM Corp.) and R version 3.4.2, Pearson's correlation coefficient were used for statistical analyses. A two-tailed $P<0.05$ was considered to indicate a statistically significant difference.

Statistical analysis. Statistical analysis was conducted using GraphPad Prism 9.5 software (Dotmatics). Data are presented as the mean ± standard deviation from at least three independent experiments. All experimental data were statistically evaluated using two-tailed unpaired Student's t-test or one-way ANOVA multiple comparison tests followed by Tukey's post hoc test. Correlation was analyzed using the Pearson's correlation coefficient, and t-distributed stochastic neighbor embedding analysis was performed to characterize the SREBP pathway genes co-expression pattern in HCC. $P<0.05$ was considered to indicate a statistically significant difference.

Results

Key genes in the SREBP pathway are dysregulated in HCC tissues compared with in normal liver tissues. The mRNA expression levels of key genes in the SREBP pathway were analyzed in HCC liver tissues and were compared with those in adjacent normal tissues using TCGA data. Specifically, SREBP2, SCAP, AMFR and SPRING were markedly upregulated, whereas PCK1 was significantly downregulated in HCC tissues (Fig. 1A). In addition, the expression levels of INSIG1, a key regulator of the SREBP pathway, were

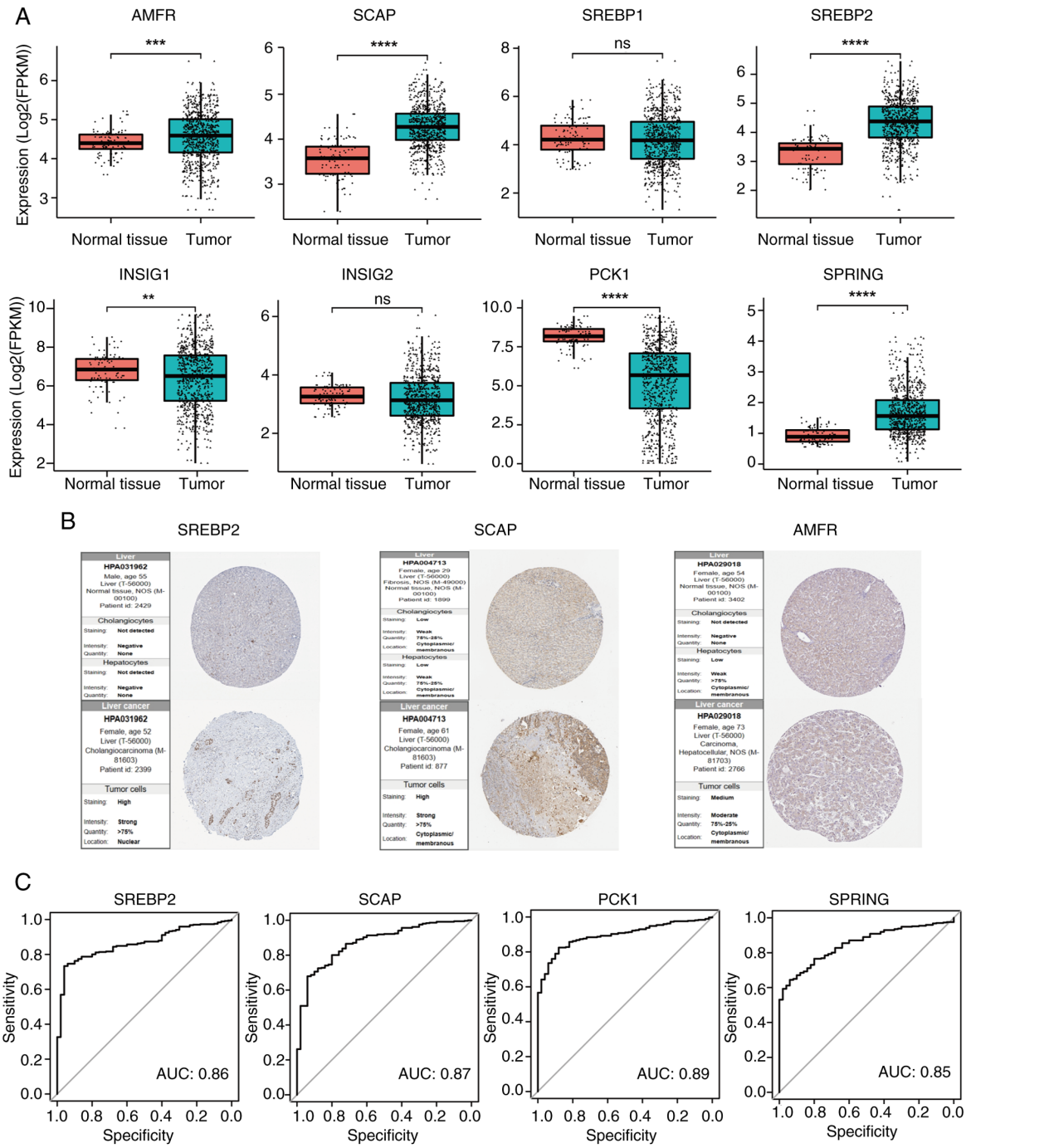

Figure 1. Key regulators of the SREBP pathway are significantly dysregulated in HCC. (A) Comparisons of mRNA expression of AMFR, SCAP, INSIG1, INSIG2, SREBP1, SREBP2, PCK1 and SPRING in HCC liver tissues and were compared with those in adjacent normal tissues using The Cancer Genome Atlas data. (B) SREBP2, SCAP and AMFR protein expression levels in HCC liver tissues and normal liver tissues based on the Human Protein Atlas (n=3). (C) Validation of the diagnostic value of key regulators of the SREBP pathway in HCC using receiver operating characteristic curves. **P<0.01, ***P<0.001, ****P<0.0001. SREBP, sterol regulatory element-binding protein; SCAP, SREBP cleavage-activating protein; INSIG, insulin-induced genes; PCK1, phosphoenolpyruvate carboxykinase 1; SPRING, SREBP regulating gene; HCC, hepatocellular carcinoma; AMFR, autocrine motility factor receptor; ns, non-significant; AUC, area under the curve.

downregulated, although another INSIG isoform, INSIG2, remained unchanged between HCC and normal tissues. To further investigate the potential effects on genes in the SREBP pathway, the NCBI GEO database was searched for relevant datasets and a study involving SCAP-knockout mice was found (GSE169104). Reanalysis of these data revealed that SCAP-knockout mice exhibited reduced expression levels of SREBP1 and SREBP2 compared with those in wild-type controls, accompanied by slightly upregulated expression of PCK1 (Fig. S1). The regulatory patterns of genes within the SREBP pathway aligned with those identified in *in vitro* tumor cell knockdown experiments, as well as expression correlation analyses derived from the clinical database findings in this study, although some differences in gene expression were noted in genes such as AMFR, SPRING, INSIG1 and INSIG2, which may be due to species-specific variations. Investigation

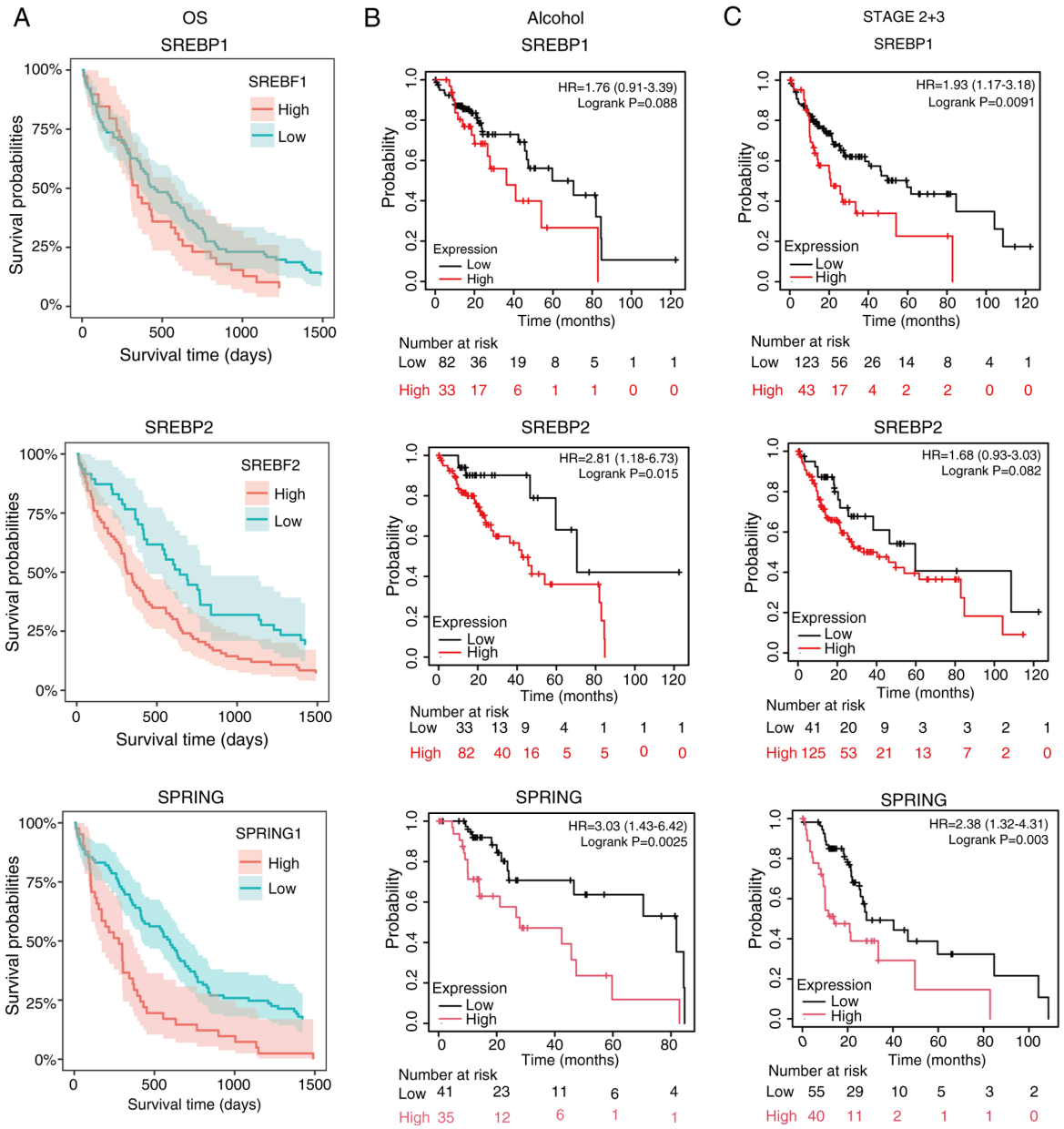

Figure 2. Kaplan-Meier survival analysis of SREBP1, SREBP2 and SPRING. (A) Patients with high expression of SREBP1, SREBP2 or SPRING had a lower OS probability compared with the low expression group. (B) Patients with high expression of SREBP1, SREBP2 or SPRING had a lower survival rate compared with the low expression group when analyzed by alcohol risk. (C) In patients with stage 2 and 3 hepatocellular carcinoma, high expression of SREBP1, SREBP2 and SPRING had a lower survival rate compared with the low expression group. SREBP, sterol regulatory element-binding protein; SPRING, SREBP regulating gene; HR, hazard ratio; OS, overall survival.

into the protein expression levels of these key SREBP pathway genes using data from the HPA database revealed a similar trend of dysregulated expression in HCC tissues, with increased expression observed for SREBP2, SCAP and AMFR (Fig. 1B). Additionally, ROC curve analysis for SREBP2, SCAP, PCK1 and SPRING demonstrated high diagnostic sensitivity for HCC, as indicated by substantial AUC values, underscoring the potential diagnostic utility of these dysregulated genes (Fig. 1C). These findings suggested that dysregulation of key genes in the SREBP pathway may occur in HCC at the mRNA and protein level.

Dysregulation of the key genes in the SREBP pathway, together with alcohol risk, predicts a poor survival rate in HCC. Patient data used in the present study were segmented into two groups, either high expression of key genes in the SREBP pathway, or low expression of key genes in the SREBP pathway. The overall survival probability of the high and low expression groups of SREBP1, SREBP2 and SPRING were assessed. The results revealed that high expression of all three genes was associated with poor survival probability (Fig. 2A). As alcohol risk affects the expression of the SREBP pathway genes in the liver (37), the survival probability in patients that consumed alcohol was also assessed. When analyzing only patients who consumed alcohol, the high expression levels of SREBP1, SREBP2 and SPRING were associated with decreased overall survival compared with low expression levels (Fig. 2B). To further investigate the association of tumor

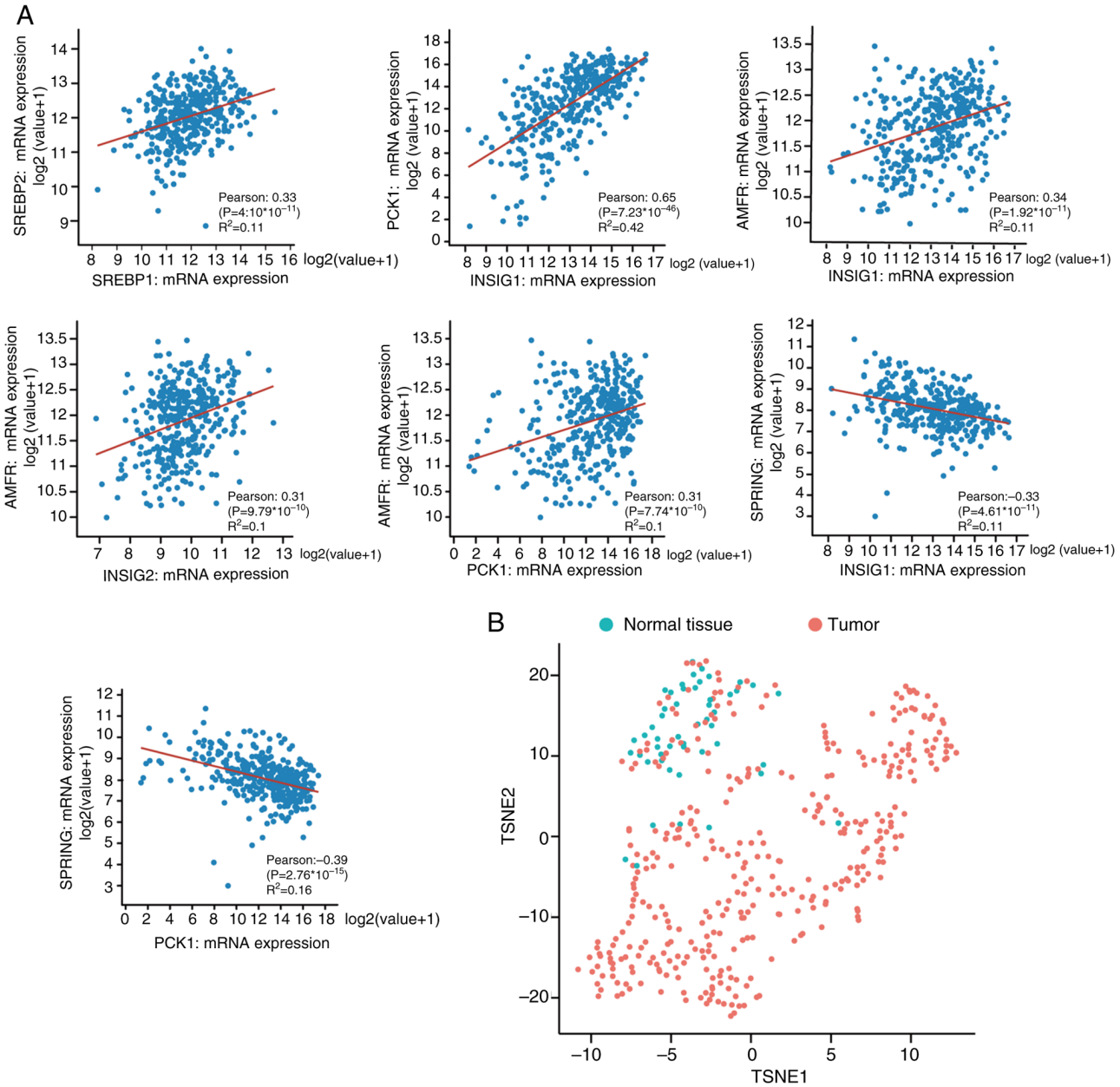

Figure 3. Correlation of the key regulators of the SREBP pathway in patients and sorting based on the expression of these genes. (A) Correlation analysis of pairs of genes in the SREBP pathway. The regression line (red) is shown (n=1,041). (B) TSNE analysis of correlation of type of samples and SREBP pathway gene expression. SREBP, sterol regulatory element-binding protein; SCAP, SREBP cleavage-activating protein; INSIG, insulin-induced genes; PCK1, phosphoenolpyruvate carboxykinase 1; SPRING, SREBP regulating gene; AMFR, autocrine motility factor receptor; TSNE, t-distributed stochastic neighbor embedding.

stage and the expression of SREBP pathway genes, overall survival was evaluated based on tumor stages. Patients with stage 2 and 3 HCC with high expression of SREBP1, SREBP2 or SPRING had decreased overall survival compared with those with low expression (Fig. 2C). These findings suggested that in the advanced stages of HCC, SREBP pathway genes could be used as a biomarker and target therapy for HCC.

SREBP pathway genes are mutually co-expressed in HCC. To further investigate the association of the expression of SREBP pathway genes in HCC tumors, co-expression of SREBP pathway genes in tumor tissues was assessed. The data were downloaded from TCGA [Liver Hepatocellular Carcinoma (TCGA, Firehose Legacy)], and the tumor samples were filtered by excluding overlapping samples and patients in this analysis. The correlation between SREBP pathway genes was assessed and the significance was quantified using the Pearson's correlation coefficient. A strong positive correlation was observed between five pairs of genes, and two pairs of genes showed significant negative correlation (Fig. 3A). SREBP1 and SREBP2 are two different SREBP isoforms, and they demonstrated positive co-expression in HCC. Similarly, AMFR interacts with the INSIG1/INSIG2 complex, and positive correlations between AMFR and INSIG1 or INSG2 were observed. As a gluconeogenic enzyme, PCK1 has been shown to phosphorylate INSIG1/2 in lipogenesis of cancer cells; in

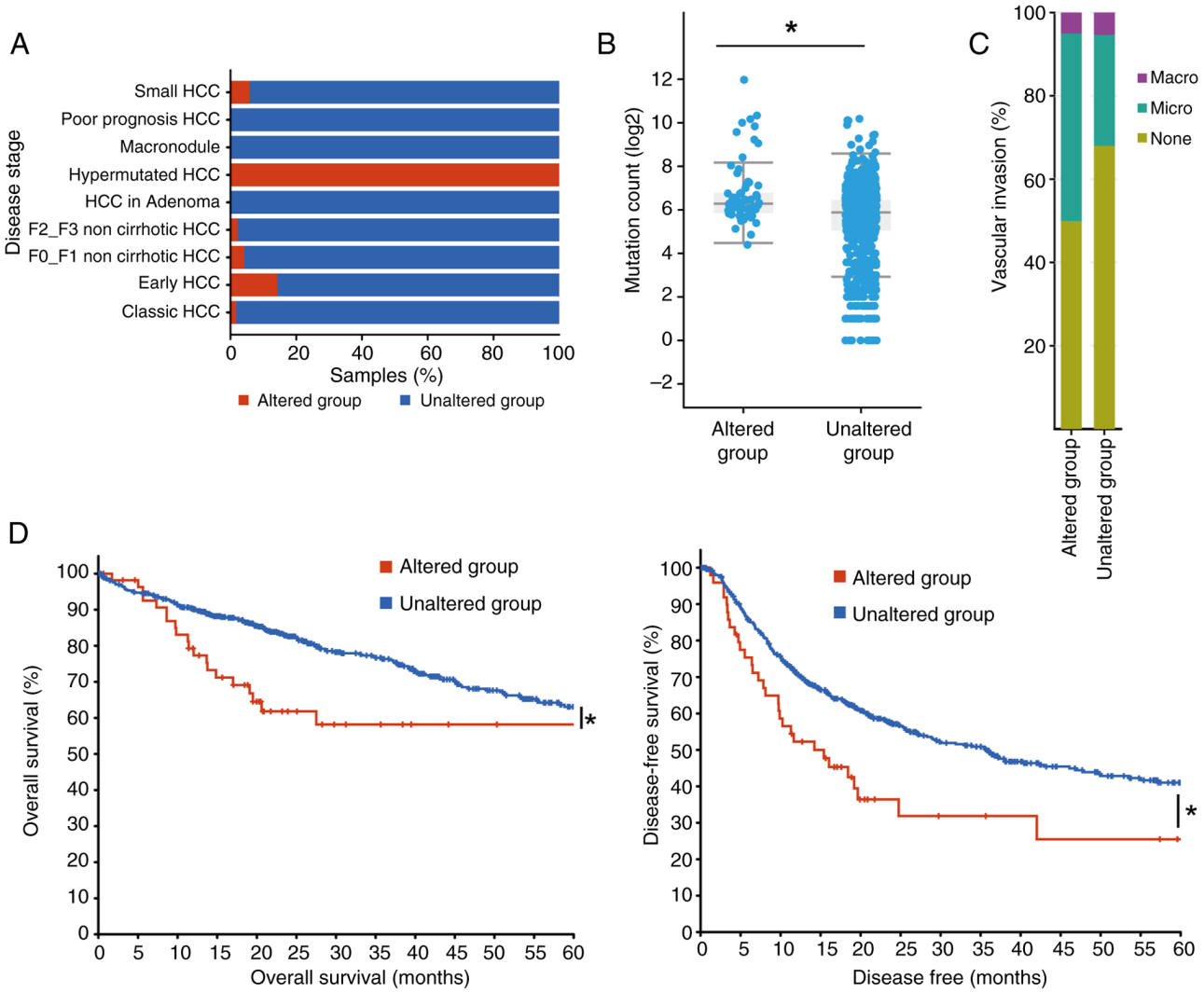

Figure 4. Clinical attributes and survival curves of the altered group of SREBP pathway regulators compared with the unaltered group. (A) Analysis of HCC stages in altered and unaltered groups. (B) Mutation count analysis of altered vs. unaltered groups. (C) Vascular invasion of the altered and unaltered groups. (D) Differences in survival rates between the altered and unaltered groups. *P<0.05. SREBP, sterol regulatory element-binding protein; HCC, hepatocellular carcinoma.

the present study (20), the co-expression of PCK1 with SREBP pathway genes was assessed. PCK1 strongly associated with INSIG1 in HCC (Pearson=0.65) and strongly co-expressed with AMFR. The correlation of SPRING expression, a potential SREBP pathway regulator, with other key factors in HCC development was also investigated. SPRING mRNA expression was strongly negatively associated with INSIG1 and PCK1. Analysis of co-expression among SREBP pathway genes in HCC tumors revealed a distinct co-expression pattern compared to normal tissues (Fig. 3B), suggesting a complex regulatory network within the pathway that may contribute to HCC progression (Fig. 3A and B).

Dysregulation of SREBP pathway group genes is associated with tumor progression in HCC. To characterize the association of the whole SREBP pathway with HCC progression, 1,043 samples from three studies (33-35) and TCGA, Firehose Legacy database in cBioPortal for Cancer Genomics were used for analysis. Samples with at least one alteration in the SREBP pathways genes, SREBP1, SREBP2, INSIG1, INSIG2, AMFR, SCAP, PCK1 and SPRING, were grouped together and labeled the 'altered group', while samples without any alterations in the SREBP pathway genes were labeled the 'unaltered group'. When stratifying patients by HCC stage, hypermutated HCC cases were predominantly associated with the altered group (Fig. 4A), and the mutation counts were significantly higher in the altered group compared with those in the unaltered group (Fig. 4B).Comparative analysis of HCC samples based on SREBP pathway gene alterations revealed a strong association with more aggressive tumor characteristics and poorer clinical outcomes, highlighting the role of the pathway in tumor progression (Fig. 4A and B). Moreover, the percentage of microvascular invasion in altered group samples was increased compared with that in the unaltered group (Fig. 4C). The survival rate of the two groups was also analyzed, which revealed that not only the overall survival rate, but also the disease-free survival rate, was significantly reduced in the altered group when compared with the unaltered group (Fig. 4D). These results suggested that alterations in the expression of any of

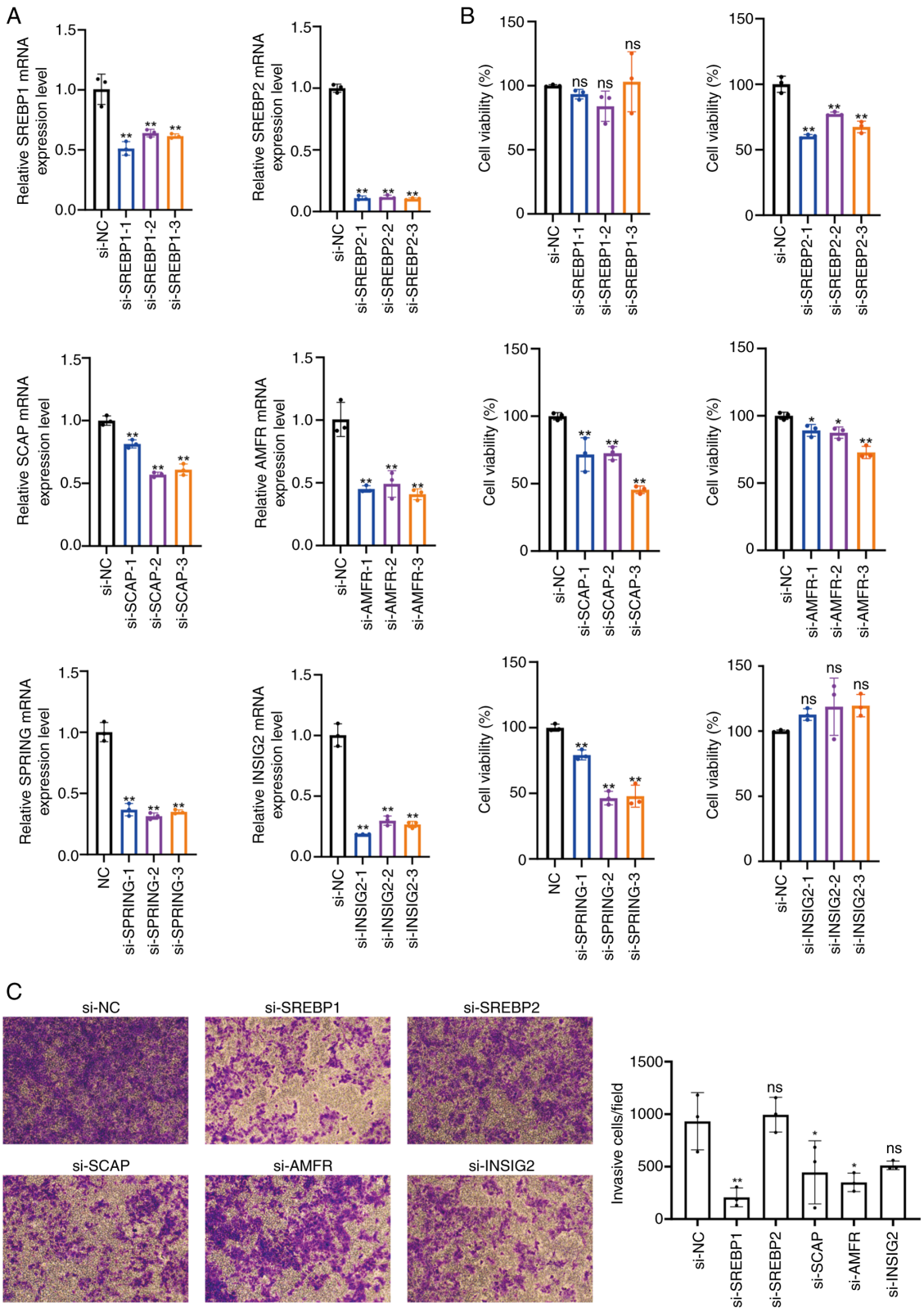

Figure 5. Genes of the SREBP pathway regulate the viability and invasion of Huh-7 cells. (A) Reverse transcription-quantitative PCR analysis verified the mRNA levels of SREBP1, SREBP2, SCAP, AMFR, SPRING and INSIG2 in Huh-7 cells after transfection with siRNAs. (B) Cell Counting Kit-8 assay was used to examine the effect of SREBP1, SREBP2, SCAP, AMFR, SPRING and INSIG2 silencing on the viability of Huh-7 cells. (C) Cell invasion was detected by Transwell invasion assay (magnification x10) and a histogram was plotted to represent the number of invasive cells. *P<0.05, **P<0.01 vs. si-NC. SREBP, sterol regulatory element-binding protein; SCAP, SREBP cleavage-activating protein; INSIG, insulin-induced genes; SPRING, SREBP regulating gene; AMFR, autocrine motility factor receptor; ns, non-significant; NC, negative control.

the investigated genes in the SREBP pathway were consistent with unfavorable overall and disease-free survival.

SREBP pathway silencing inhibits the viability and invasion of HCC cells. Next, the impact of SREBP pathway genes

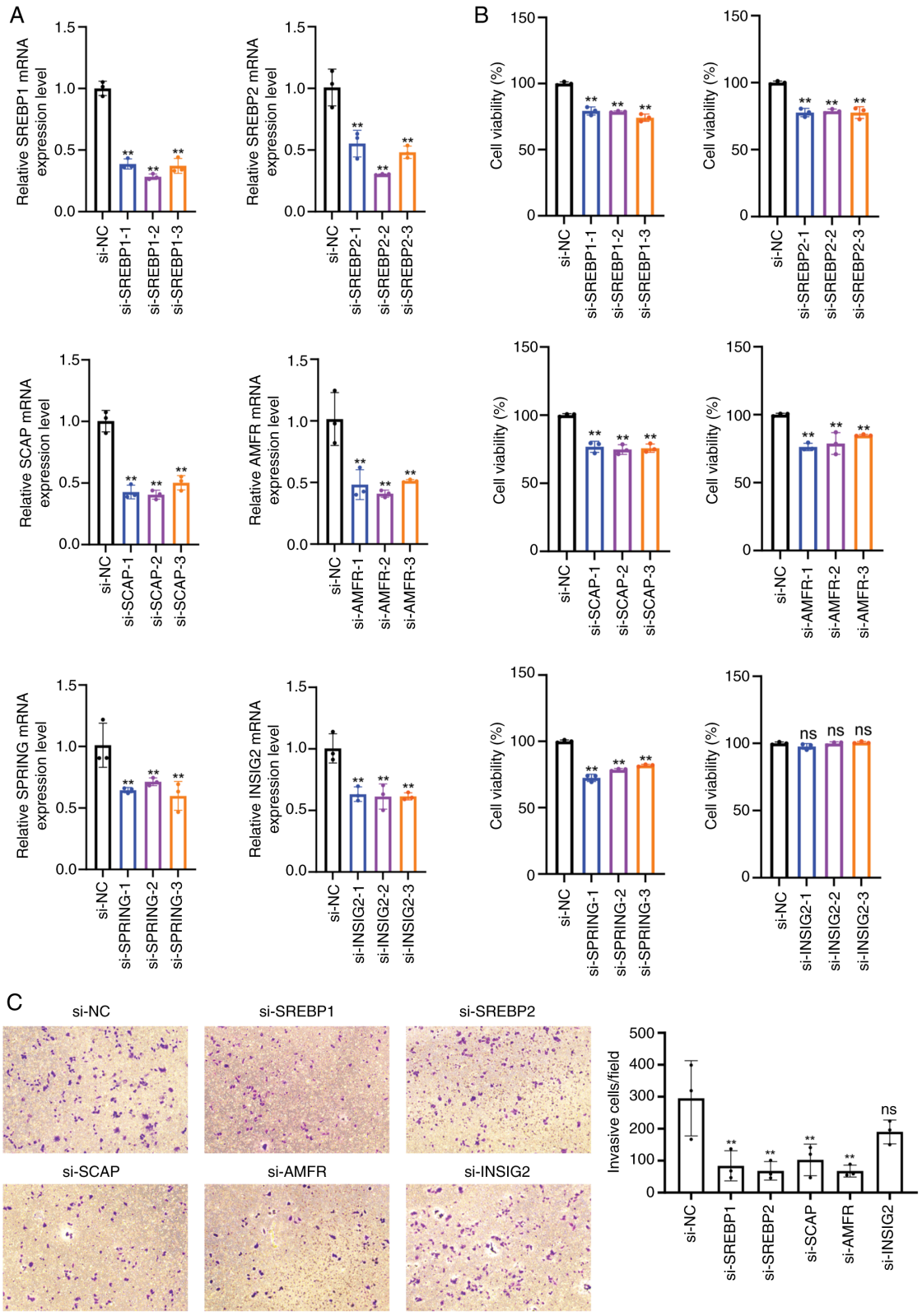

Figure 6. SREBP pathway genes regulate the viability and invasion of HCC-LM3 cells. (A) Reverse transcription-quantitative PCR analysis verified the mRNA levels of SREBP1, SREBP2, SCAP, AMFR, SPRING and INSIG2 in HCC-LM3 cells after transfection with siRNAs. (B) Cell Counting Kit-8 assay was used to examine the effect of SREBP1, SREBP2, SCAP, AMFR, SPRING and INSIG2 silencing on the viability of HCC-LM3 cells. (C) Cell invasion was detected by Transwell invasion assay (magnification, x10) and a histogram represented the number of invasive cells. **P<0.01 vs. si-NC. SREBP, sterol regulatory element-binding protein; SCAP, SREBP cleavage-activating protein; INSIG, insulin-induced genes; SPRING, SREBP regulating gene; AMFR, autocrine motility factor receptor; NC, negative control.

on the behavior of HCC cells was investigated. Specifically, three siRNAs targeting SREBP1, SREBP2, SCAP, AMFR, SPRING and INSIG2 were individually transfected into Huh-7 cells. RT-qPCR analysis revealed a significant decrease

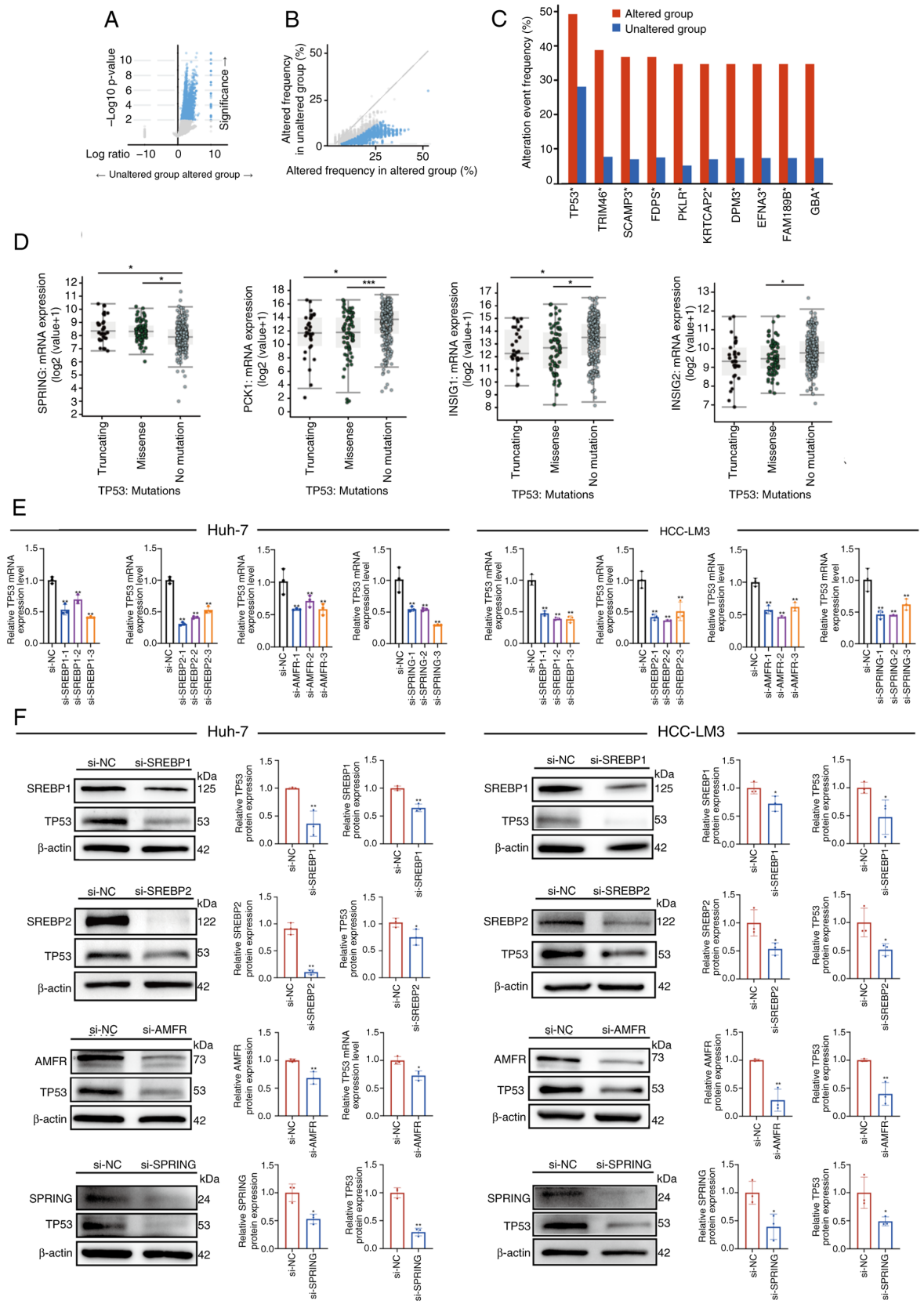

Figure 7. Expression of the SREBP pathway genes may be associated with TP53 mutations in HCC. (A) Volcano plot of mutations between altered and unaltered group samples. Samples in the upper left and right quadrants contain significantly differentially expressed mutations. (B) Volcano plot of altered frequency in different group samples. (C) Top 10 significantly mutated genes between the altered and unaltered groups. The alteration event frequency was analyzed. (D) Comparison of mRNA expression levels of SREBP pathway genes, SPRING, PCK1, INSIG1 and INSIG2, between the TP53 mutation groups and no mutation group. (E) Reverse transcription-quantitative PCR analysis of the mRNA levels of TP53 in Huh-7 and HCC-LM3 cells with SREBP1, SREBP2, AMFR and SPRING knockdown, compared with si-NC cells. (F) Western blot analysis was carried out to examine SREBP1, SREBP2, AMFR, SPRING and TP53 protein expression levels, compared with those in the si-NC-transfected Huh-7 and HCC-LM3 cells. *P<0.05, **P<0.01, ***P<0.001 vs. si-NC or as indicated. SREBP, sterol regulatory element-binding protein; SCAP, SREBP cleavage-activating protein; INSIG, insulin-induced genes; SPRING, SREBP regulating gene; AMFR, autocrine motility factor receptor; NC, negative control.

in the mRNA expression levels of these genes in Huh-7 cells upon siRNA transfection (Fig. 5A). Upon silencing of SREBP2, SCAP, AMFR and SPRING, a considerable reduction in cell viability was observed, whereas SREBP1 and INSIG2 silencing did not affect cell viability (Fig. 5B). Additionally, the knockdown of SREBP1, SCAP and AMFR resulted in significant inhibition of Huh-7 cell invasion, while the knockdown of SREBP2 and INSIG2 showed no significant difference when compared with the control (Fig. 5C). These findings suggested that silencing SREBP2, SCAP, AMFR and SPRING, which are the components of the SREBP pathway, may impede Huh-7 cell viability, whereas silencing SREBP1, SCAP and AMFR could inhibit the invasion of Huh-7 cells. In HCC-LM3 cells, knocking down the SREBP pathway genes, except for INSIG2, invariably and significantly reduced cell viability and invasion (Fig. 6A-C). These results indicated the role of this pathway in influencing the behavior of HCC cells.

SREBP pathway alteration is associated with mutations in the TP53. Genomic analysis between groups with and without SREBP pathway alterations revealed an increased frequency of oncogenic mutations in the altered group, suggesting an association between SREBP pathway dysregulation and the genetic landscape of HCC (Fig. 7A and B). The frequency of alteration events of TP53 were ~49.25% in the altered group and 28.1% in the unaltered group (Fig, 7C). TP53 mutations were grouped into missense and truncated, and analysis of these groups revealed that they exhibited significantly increased expression of SPRING, and reduced expression of PCK1 and INSIG1 when compared with the no mutations in TP53 group (Fig. 7D). PCK1 and INSIG1 are negative regulators of SREBPs (12,13,20). Levels of INSIG2 were significantly decreased in the missense group but not in the truncated group when compared with the no mutation group (Fig. 7D). Additionally, to investigate the relationship between the SREBP pathway and TP53, SREBP1, SREBP2, AMFR and SPRING were knocked down in Huh-7 and HCC-LM3 cells using siRNAs. The mRNA and protein levels of TP53 were decreased in response to silencing of the various SREBP pathway genes when compared with the control group (Fig. 7E and F). Hence, mutated TP53 might be associated with SREBP pathway dysregulation. Overall, these data indicated that SREBP pathway gene dysregulation may be associated with TP53 mutations, potentially resulting in the establishment and progression of HCC.

Discussion

The SREBP pathway, a pivotal signaling cascade in lipid metabolism, has been implicated in the regulation of tumor proliferation and metastasis through *in vitro* and *in vivo* mouse experiments (38,39). Prior studies have established the role of individual genes in HCC; however, the collective function of SREBP pathway genes in the diagnosis and treatment of HCC remains inadequately investigated (17,18). The findings of the present study suggested that a combinatorial assessment of individual SREBP pathway gene expression patterns could offer new potential avenues for early-intermediate stage diagnosis and therapeutic intervention in HCC. Moreover, the present study provides evidence that the SREBP pathway holds potential as a prognostic biomarker in HCC.

As master regulators of transcription, SREBPs orchestrate the expression of genes essential for the biogenesis of cholesterol, fatty acids and triglycerides (10,11). Their dysregulation is associated with dyslipidemia, diabetes mellitus, non-alcoholic fatty liver disease, non-alcoholic steatohepatitis (NASH), liver fibrosis, chronic kidney disease, neurodegenerative diseases and various types of cancer (40). In metabolic dysfunction-associated fatty liver disease, SREBP-1c is persistently activated, driving increased lipid synthesis, which contributes to the progression of hepatic steatosis. SREBP2 has also been associated with liver fibrosis by regulating cholesterol levels in hematopoietic stem cells (17), suggesting its potential role as an early biomarker for liver dysfunction and HCC progression.

Other than HCC, SREBPs have also been implicated in the lipid metabolic reprogramming of other types of cancer, such as breast cancer, prostate cancer and glioblastoma (41). In breast cancer, the activation of SREBP is associated with poor prognosis, while SREBP2 upregulation is observed in prostate cancer, highlighting the role of SREBPs in the metabolic reprogramming of various malignancies (41). In neurodegenerative diseases, such as Alzheimer's disease, altered lipid homeostasis regulated by SREBPs may exacerbate neurodegeneration (40), suggesting a potential role for SREBP pathway genes as biomarkers in these disorders. Similarly, increased SREBP activity in immune cells contributes to the production of inflammatory lipids, exacerbating conditions such as rheumatoid arthritis and systemic lupus erythematosus (42,43), indicating that SREBPs may also serve as biomarkers for inflammatory diseases.

Despite its established importance, the clinical implications of the SREBP pathway, particularly its role in the progression of HCC, remain poorly investigated. The findings of the present study revealed a significant upregulation of the majority of genes involved in the canonical SREBP pathway in HCC tissues, with the exception of PCK1, a negative regulator of the SREBP pathway (20), which was found to be significantly downregulated. These alterations at the mRNA and protein levels suggest a potential dysregulation of lipid metabolism in HCC. Additionally, the diagnostic utility of these genes was supported by ROC curves, indicating their potential as biomarkers for the molecular diagnosis of HCC. Multiple steps of SREBP activation form complex regulatory networks. The analysis of the present study extended beyond gene expression, incorporating survival probabilities alongside alcohol risk and tumor stages. Co-expression analysis further emphasized the complexity of this pathway, revealing that a combination of gene expression patterns could offer a more accurate assessment of HCC progression. The findings of the present study expanded the understanding of the SREBP pathway by including both key and newly identified regulators. The present study revealed that dysregulation within this pathway was associated with adverse clinical outcomes such as vascular invasion, poor survival rates and advanced tumor stages. Furthermore, the effects of silencing key genes of the SREBP pathway were assessed, which revealed the viability and invasion of HCC cells were reduced. Among the dysregulated genes, TP53 emerged as a significant factor, suggesting

its role in SREBP pathway modulation and presenting a new target for exploring oncogenic mechanisms.

Several studies have shown that SREBPs are associated with different types of cancer, such as colorectal cancer, prostate cancer, breast cancer, endometrial carcinoma and nasopharyngeal carcinoma (39,44-47). In most types of cancer, SREBPs are overexpressed in human tumors compared with in normal tissues. Likewise, studies in patients with HCC have demonstrated that SREBP1 is elevated in tumor tissue (48,49). Consistent with these findings, the present study showed that SREBP2 was significantly upregulated in HCC. By contrast, the mRNA levels of SREBP1 did not exhibit differences between tumor and normal tissues. The discrepancy may stem from the use of a large patient cohort in the present study, compared with those used in previous analyses (48,49), which showed SREBP-1 was expressed at higher levels in patients with large tumor size and were based on a smaller database (n<50 samples). Benefiting from the large database, the present study demonstrated that not only the levels of key transcription factors of the SREBP pathway were associated with tumor progression, but other key genes and regulators including SCAP, INSIG1, AMFR, PCK1 and SPRING were also associated with it. Furthermore, alcohol risk, and tumor stage were addressed. Genes of the SREBP pathway could serve as potential biomarkers or parameters to improve clinical decisions. Considering the diversity of clinical cases, a combination of two or more gene expression patterns of the SREBP pathway might provide improved results for diagnosis and prognostic evaluation.

A previously unrecognized factor, SPRING, was identified as a determinant of SREBP signaling by regulating SCAP (21). In the current study, the role of SPRING in HCC progression was thoroughly investigated. Among all the regulatory genes of the SREBP1/2 pathways, the present study revealed that only high SPRING expression, in association with alcohol consumption and advanced tumor stages, was significantly linked to poor survival probability (stage 2 and 3 of HCC), highlighting its potential as a specific biomarker for more aggressive forms of the disease. Moreover, SPRING was revealed to be negatively associated with other previously identified regulators of the SREBP pathway, PCK1 and INSIG1, which further qualified the study of the underlying mechanism of the SREBP signaling pathway in HCC progression. In addition to this, epidemiological studies have shown that 25% of cirrhotic livers induced by NASH eventually progress to HCC. Our unpublished data showed that SPRING was highly upregulated in patients with NASH, which may further accelerate HCC progression.

In the present study, by comparing the altered and unaltered group samples, a considerable number of dysregulated genes were identified. The top dysregulated gene was TP53, which is demonstrated to mediate SREBP2 maturation during tumor progression (50). In line with this evidence, the present study highlighted a high incidence of TP53 mutations within the dysregulated SREBP pathway gene expression cohort. TP53 mutants exhibit a dual role by losing their tumor-suppressive function and acquiring oncogenic properties (51). The findings of the present study revealed that the downregulation of genes within the SREBP pathway led to a reduction in TP53 expression levels. This downregulation also inhibited the viability and invasive capabilities of the TP53-mutant HCC cell lines, Huh-7 and HCC-LM3. These findings underscore a potential interplay between the SREBP pathway and TP53 mutants, suggesting a bidirectional regulation mechanism. Additionally, tripartite motif TRIM46 emerged as the second most frequently mutated gene in the analysis. Several TRIM family members, such as TRIM31 and TRIM32, have previously been identified as oncogenes in HCC (52,53). Notably, the TRIM family of genes tend to be upregulated in TP53-mutant tumors and are associated with the activation of cell cycle-related genes, particularly in the context of TP53 mutations (54). Furthermore, other mutated genes were identified between the altered and unaltered groups, including EFNA3 and FAM189B, which are associated with tumor progression and poor prognosis in patients with HCC (55,56). Building upon these observations, further investigations are warranted to elucidate how the SREBP pathway modulates liver tumorigenesis through the regulation of oncogene expression pathways.

In summary, the genes in the SREBP pathway were significantly dysregulated in HCC tissues compared with those in normal liver tissues. Moreover, the dysregulation of these genes was associated with tumor progression and predicted an unfavorable survival rate. Furthermore, the individual genes in the SREBP pathway were co-expressed and were associated with TP53 mutations. Combination evaluation of the expression levels of several genes may provide a new strategy for HCC diagnosis.

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Availability of data and materials

The data generated in the present study may be requested from the corresponding author.

Authors' contributions

XL, YW, JL, TG, LC, MY and NL contributed to the design, data analysis and review of the manuscript. NL conducted conceptualization, methodology and wrote the manuscript. MY conducted bioinformatics analyses and critically edited the manuscript. XL and NL confirm the authenticity of all the raw data. All authors read and approved the final version of the manuscript.

Ethics approval and consent to participate

Not applicable.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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