Squalene epoxidase promotes hepatocellular carcinoma development by activating STRAP transcription and TGF-β/SMAD signaling

Wu Yin[1], zhirui zhang[2], Wei Wu[3], Hao Jiao[4], Yuzhong Chen[5], Xiaojun Ji[6], Jing Cao[7], and Fangzhou Yin[8]

[1]Nanjing University
[2]The State Key Lab of Pharmaceutical Biotechnology, College of Life Sciences, Nanjing University, Nanjing
[3]Organ Transplantation Center, Southern District, the First Affiliated Hospital of University of Science and Technology of China (Anhui Provincial Hospital)
[4]Department of Pharmacy, Fuyang People's Hospital
[5]Department of Surgical OncologyFirst Affiliated Hospital of Bengbu Medical College
[6]Department of Innovation, Nanjing Chia Tai Tianqing Pharmaceutical Co., Ltd
[7]Department of Pharmacy, Women's Hospital of Nanjing Medical University/Nanjing Maternity and Child Health Care Hospital
[8]School of Pharmacy, Nanjing University of Chinese Medicine

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Abstract

Background and Purpose Squalene epoxidase (SQLE) is a key enzyme involved in cholesterol biosynthesis, but increasing evidence reveals that SQLE is abnormally expressed in some types of malignant tumors, and the underlying mechanism remains poorly understood. Experimental Approach Bioinformatics analysis and RNA sequencing were applied to detect to differentially expressed genes in clinical HCC tumors. AnnexinV/PI, EdU assay, transwell, western blot, qRT-PCR, IHC staining, RNA sequencing, dual-luciferase reporters and HE staining were evaluated to investigate the pharmacological effects and possible mechanisms of SQLE in vitro and in vivo. Key Results We found that SQLE expression is specifically elevated in HCC tumors, correlating with poor clinical outcomes. SQLE promoted HCC growth, EMT, and metastasis both in vitro and in vivo. In contrast, silencing of SQLE expression prevented HCC development. Both RNA-seq and functional experiments revealed that the protumorigenic effect of SQLE on HCC is closely related to the activation of cellular TGF-β/SMAD signaling, but interestingly, the stimulatory effect of SQLE on TGF-β/SMAD signaling and HCC development is also critically dependent on STRAP. SQLE expression is well correlated with STRAP in HCC, and further, to amplify TGF-β/SMAD signaling, SQLE even transcriptionally increased STRAP expression mediated by the trans-acting factor AP-2α. Finally, as a chemical inhibitor of SQLE, NB-598 markedly inhibited HCC cell growth and tumor development in mouse models. Conclusions and Implications Taken together, SQLE serves as a novel oncogene in HCC development by activating TGF-β/SMAD signaling, and targeting SQLE could be useful in drug development and therapy for HCC.

Σχυαλενε εποξιδασε προμοτες ηεπατοχελλυλαρ χαρχινομα δεελοπμεντ βψ αχτιατινγ ΣΤΡΑΠ τρανσχριπτιον ανδ ΤΓΦ-β/ΣΜΑΔ σιγναλινγ

Zhirui Zhang*[1], Wei Wu*[2], Hao Jiao[3], Yuzhong Chen[4], Xiaojun Ji[5], Jing Cao[6], Fangzhou Yin[7], and Wu Yin[1]

[1] The State Key Lab of Pharmaceutical Biotechnology, College of Life Sciences, Nanjing University, Nanjing, 210046, China.

[2] Organ Transplantation Center, Southern District, the First Affiliated Hospital of University of Science and Technology of China (Anhui Provincial Hospital), Hefei, 230001, China

[3] Department of Pharmacy, Fuyang People's Hospital, Fuyang, 236000, China

[4] Department of Surgical Oncology,First Affiliated Hospital of Bengbu Medical College,Bengbu, 233000, China

[5] Department of Innovation, Nanjing Chia Tai Tianqing Pharmaceutical Co., Ltd,Nanjing, 210000, China

[6] Department of Pharmacy, Women's Hospital of Nanjing Medical University/Nanjing Maternity and Child Health Care Hospital, Nanjing, 210004, China

[7] School of Pharmacy, Nanjing University of Chinese Medicine, Nanjing, 210046, China

[*] These authors contributed equally to this work.

Correspondence to:

(1) Yin Wu Ph.D.

State Key lab of Pharmaceutical Biotechnology, College of Life Sciences in Nanjing University (Xianlin Campus), Nanjing, 210046, China

Email address: wyin@nju.edu.cn

(2) Jing Cao

Department of Pharmacy, Women's Hospital of Nanjing Medical University/Nanjing Maternity and Child Health Care Hospital, Nanjing, 210004, China

E-mail address: *njfycj@163.com*

(3) Yin Fang-zhou Ph.D.

School of Pharmacy, Nanjing University of Chinese Medicine, Nanjing, 210046, China

E-mail address; yfz2003@163.com

Abstract

Background and Purpose

Squalene epoxidase (SQLE) is a key enzyme involved in cholesterol biosynthesis, but increasing evidence reveals that SQLE is abnormally expressed in some types of malignant tumors, and the underlying mechanism remains poorly understood.

Experimental Approach

Bioinformatics analysis and RNA sequencing were applied to detect to differentially expressed genes in clinical hepatocellular carcinoma (HCC) tumors. MTT, colony formation assay, AnnexinV-FITC/PI, EdU assay, wound healing, transwell, western blot, qRT-PCR, IHC staining, cytoskeleton F-actin filaments assay, RNA sequencing, dual-luciferase reporters and HE staining were evaluated to investigate the pharmacological effects and possible mechanisms of SQLE in vitro and in vivo.

Key Results

We found that SQLE expression is specifically elevated in hepatocellular carcinoma (HCC) tumors, correlating with poor clinical outcomes. SQLE significantly promoted HCC growth, epithelial-mesenchymal transition, and metastasis both in vitro and in vivo. In contrast, silencing of SQLE expression prevented

HCC development. Both RNA-seq and functional experiments revealed that the protumorigenic effect of SQLE on HCC is closely related to the activation of cellular TGF-β/SMAD signaling, but interestingly, the stimulatory effect of SQLE on TGF-β/SMAD signaling and HCC development is also critically dependent on STRAP, a serine and threonine kinase. SQLE expression is well correlated with STRAP in HCC, and further, to amplify TGF-β/SMAD signaling, SQLE even transcriptionally increased STRAP gene expression mediated by the trans-acting factor AP-2α. Finally, as a chemical inhibitor of SQLE, NB-598 markedly inhibited HCC cell growth and tumor development in mouse models.

Conclusions and Implications

Taken together, SQLE serves as a novel oncogene in HCC development by activating TGF-β/SMAD signaling, and targeting SQLE could be useful in drug development and therapy for HCC.

Abbreviations

HCC, hepatocellular carcinoma; SQLE, squalene epoxidase; NAFLD, nonalcoholic fatty liver disease; AFP, alpha fetoprotein; EdU, 5-ethynyl-2'-deoxyuridine; DEGs, differentially expressed genes; OS, overall survival; RFS, recurrence-free survival; PFS, progression-free survival; DSS, disease-specific survival; EMT: epithelial-mesenchymal transformation; STRAP, serine-threonine kinase receptor-associated protein

Keywords : squalene epoxidase; hepatocellular carcinoma; TGF-β/SMADs; STRAP; NB-598

What is already known

a. NB-598 is an inhibitor of SQLE.

b. The knowledge of SQLE in HCC is laconic with an unclarified mechanism

What this study adds

a. SQLE is highly expressed in HCC clinical samples and correlates with poor prognosis.

b. SQLE promotes HCC development by activating STRAP transcription and TGF-β/SMAD signaling.

c. SQLE transcriptionally upregulates STRAP expression in HCC cells by trans-acting factor AP-2α.

Clinical significance

SQLE is a promising target for HCC therapy and drug development.

Introduction

Hepatocellular carcinoma (HCC) has developed into the third leading cause of tumor-related death and has become the sixth most common tumor in the world (Sung *et al.* , 2021; Zhang *et al.* , 2021). According to statistics, there were 905,677 new liver cancer patients worldwide in 2020 and 830,180 new liver cancer deaths(Sung *et al.* , 2021). Hepatitis viral infection, aflatoxin, alcohol, and lipogenesis are the main risk factors during the HCC formation process (Erkekoglu *et al.* , 2017; Chen *et al.* , 2019). Although the diagnosis and treatment methods of HCC have always been developing, surgical resection and liver transplantation are still the most effective treatments for HCC. As a highly aggressive tumor, venous invasion and intrahepatic and distant metastasis often occur in the process of HCC, which is the main cause of high recurrence and mortality (Yang *et al.* , 2019a; Chen *et al.* , 2020). Therefore, it is very important to explore new therapeutic and diagnostic targets for HCC.

Metabolic abnormalities are one of the hallmarks of tumor cells(Counihan *et al.* , 2018). Reports have shown that cholesterol metabolism in tumor cells is different from that in normal cells. In patients with breast or prostate carcinoma, serum cholesterol content is positively correlated with the risk of cancer (Kuzu *et al.* , 2016; Xu *et al.* , 2020). There are more than 30 different enzymes involved in the cholesterol synthesis pathway (Sharpe *et al.* , 2013). SQLE catalyzes the first oxidation step in sterol biosynthesis and produces two key downstream metabolites, cholesterol ester and nicotinamide adenine dinucleotide phosphate (NADP+) (Gill *et al.* , 2011; Zhang *et al.* , 2019). The gene of SQLE is located on human chromosome

8q24.1. Recent studies have found that SQLE is highly expressed in some types of malignant tumors, including prostate cancer, breast cancer, lung cancer, and colon cancer (Brown*et al.* , 2016; Stopsack *et al.* , 2016; Ge *et al.* , 2019; Jun *et al.* , 2021), suggesting that it could be a drug target for cancer therapy. Reports have also demonstrated that SQLE expression is upregulated in hepatocellular carcinoma. Nonalcoholic fatty liver disease (NAFLD) increases the risk of hepatocellular carcinoma. Notably, SQLE was the top outlier gene that was highly expressed in NAFLD-HCC patients. By increasing the production of $NADP^+$ and cholesteryl ester, SQLE epigenetically inactivated PTEN expression and promoted HCC development as a result of reactive oxygen species generation and AKT-mTOR activation. Chemical inhibitors of SQLE suppressed HCC development by reducing cholesterol content and arresting cells at G2 phase (Liu *et al.* , 2018).

Although the oncogenic role of SQLE in HCC has been increasingly appreciated, the underlying signaling mechanism remains unclear. TGF-β/SMAD signaling regulates a wide array of cellular processes in tumor development (Nickel *et al.* , 2018). Here, we described a novel role of SQLE in HCC development; that is, SQLE is a strong activator of TGF-β/SMAD signaling in HCC mediated by STRAP, a serine/threonine kinase. Moreover, TGF-β/SMAD activation by SQLE is an early event in HCC that occurs upstream of PTEN inactivation.

Materials and methods

Human samples collection

A total of 6 paired human HCC tumor and adjacent nontumor tissues were collected from patients with biopsy-proven HCC at the First Affiliated Hospital of University of Science and Technology of China (Anhui Provincial Hospital). All human HCC tissues obtained written informed consent from patients or their guardians. The Institutional Review Board of the University of Science and Technology of China approved the use of the tumor specimens in this study (2020KY131).

Reagents and antibodies

The Annexin V-FITC/PI Apoptosis Detection Kit and HifairTM II 1st Strand cDNA Synthesis SuperMix were purchased from Yeasen Biotech (Shanghai, China). DAPI and BeyoClick EdU Cell Proliferation Kit with Alexa Fluor 594 were obtained from Beyotime Institute of Biotechnology (Wuhan, China). SB-431542 was acquired from MedChemExpress (Shanghai, China). The TraKine^{TM}F-actin Staining kit (Green Fluorescence) was purchased from Abbkine Scientific (Wuhan, China). Alpha fetoprotein (AFP) ELISA was acquired from NEOBIOSCIENCE (Shenzhen, China). NB-598 was obtained from MedMol (Shanghai, China). The following antibodies were used: SQLE, STRAP, DDDDK-tag, GAPDH and Tubulin α (Bioworld, Nanjing, China); PCNA, E-cadherin, N-cadherin, Vimentin, SMAD3, SMAD2 and HA-Tag (Proteintech, Wuhan, China); phospho-SMAD2 (Affinity Biosciences, OH, USA); phospho-SMAD3 (Abcam, Cambridge, MA, USA); and TGF-β1 (Zen Bioscience, Chengdu, China).

Cell lines and cell culture

The HEK-293T and hepatocellular carcinoma cell lines SMMC7721, Huh-7, and H22 were all obtained from Shanghai Cell Bank (Shanghai, China). Cells were maintained in DMEM (WISENT, Canada) supplemented with 10% fetal bovine serum (FBS) (ExCell Bio, Shanghai, China), 100 U/mL penicillin, and 100 μg/mL streptomycin. Cells were cultured at 37 °C with 5% CO_2.

Cell transfection

The cells were transfected with the plasmids using Hieff Trans^{TM} Liposomal Transfection Reagent (Yeasen Biotech, Shanghai, China) and transfected with siRNA with Lipofectamine 2000 Transfection Reagent (Thermo Fisher, Waltham, MA, USA) according to the manufacturer's instructions. The following siRNAs were used in this study: SQLE siRNA#1: GAAACAAGCUUAAUAGGAATT; SQLE siRNA#2: CCAAGGAAUUUAAGA

GAAUTT; STRAP siRNA: AGAAAUUGCUUCAGAGAAUTT; SP1 siRNA: GGAUGGUUCUGGUCAAAUATT; CEBP/β siRNA: AGGUCAAGAGCAAGGCCAATT; AP-2α siRNA: CGCCAAAAGCAGUGACAAATT, and the control siRNA was UUCUCCGAACGUGUCACGUTT.

Western blot analysis

Cells were harvested and homogenized in RIPA lysis buffer (Beyotime Institute of Biotechnology), and the protein concentration was detected by BCA assay (Yeasen Biotech, Shanghai, China). Equal amounts of total protein were separated by SDS–PAGE and transferred to PVDF membranes. After blocking with 5% nonfat milk for 2 h, the membranes were incubated with specific primary antibodies for at least 4 h, followed by incubation with horseradish peroxidase-conjugated anti-rabbit or anti-mouse IgG for 1 h at room temperature. All membranes were imaged with ECL super (Sparkjade, Shandong, China) and acquired by Tanon 4600SF (Shanghai, China).

RNA isolation and real-time quantitative PCR (qRT–PCR)

Total RNA was extracted using SparkZol Reagent (Shandong Sparkjade Biotechnology Co., Ltd., Shandong, China) according to the manufacturer's instructions. qRT–PCR was performed using the SYBR® Green Premix Pro Taq HS qPCR Kit (Accorate Biology, Hunan, China). The primer sequences are listed in Supplementary Table S1.

Immunohistochemistry (IHC) staining

IHC staining was performed as previously reported (Yang *et al.* , 2019b). In brief, paraffin sections were deparaffinized with xylene and rehydrated with graded ethanol solutions (100%, 95%, 80%). Hydrogen peroxide (3%) was used to quench endogenous peroxidase activity, and the sections were incubated for 10˜15 min. Then, the sections were microwaved for 20 min in a citric acid solution (pH=6.0) at 95 °C and blocked with 5% BSA for 30 min after cooling. Primary antibodies against SQLE (BS71537, 1:200), PCNA (10205-2-AP, 1:500), SMAD2 (12570-1-AP, 1:300), and SMAD3 (25494-1-AP, 1:300) were incubated overnight. After washing three times with PBS, the sections were incubated with a horseradish-conjugated secondary antibody for 1 h, followed by DAB substrate kit (Boster, Wuhan, China) display signal. The images were all captured with an optical microscope (NEXCOPE930, Nexcope, USA).

Cell viability assays

The cells were plated at a density of 5×10^3 cells/well in 96-well plates. After treatments, 15 μl of 5 mg/ml MTT was added, and then the plates were incubated further for 2 h at 37 °C. After supernatant removal, 150 μl DMSO was added to each well, and the absorbance of each sample was read at 490 nm using a spectrophotometer (Elx800, BioTEk instrument, USA).

Colony formation assay

Cells at a density of 3×10^3/ml were seeded in a 12-well plate and cultured continuously until colonies were formed after plasmid transfection. Then, the cells were fixed with paraformaldehyde for at least 10 min and stained with 1% crystal violet for 10˜15 min. After washing twice with PBS, cell colonies were photographed using a camera.

Annexin V-FITC/PI apoptosis assay

The cells (1.5×105 cells/well) were seeded in a 12-well plate and transfected with siNC or siSQLE for 36 h. Cells were harvested and analyzed by flow cytometry using an Annexin V-FITC/PI apoptosis detection kit. Before measurement, cells were incubated in buffer containing 400 μl of Annexin-binding buffer, 5 μl of Annexin V-FITC, and 10 μl PI for 30 min in the dark. Flow cytometry analysis was conducted on a BD Accuri C6 flow cytometer (BD Biosciences, San Jose, USA).

5-ethynyl-2'-deoxyuridine (EdU) assay

An EdU incorporation assay was performed using the BeyoClick EdU Cell Proliferation Kit with Alexa Fluor 594 according to the manufacturer's instructions. Briefly, cells after treatments were seeded into 24-well plates at a density of 1.5 $\times 10^5$ cells/well and then incubated with 10 μM EdU for 2 h at 37 °C. The medium was removed, and samples were fixed with 4% paraformaldehyde for 15 min and permeabilized with 0.3% Triton X-100 for another 15 min. After washing with PBS, the cells were reacted with 1× Click reaction cocktail for 30 min in the dark, and the nuclei were stained with Hoechst 33342 for 10 min. Images were captured with an optical microscope.

Wound healing assays

Huh7 or SMMC7721 cells (1.5 $\times 10^5$ cells/well) were seeded in 12-well plates. After transfection for 24 h, cell wounds were made using a 100 μl pipette tip (24 h after transfection), and cells were further cultured in fresh medium for 36 h. The wound healing of each sample was observed at 0 and 36 h and captured by an inverted optical microscope.

Transwell migration assay

After transfection for 24 h, Huh7 or SMMC7721 cells at a density of 2×10^4 cells/well were seeded into the upper chamber in serum-free medium, and the bottom chamber was loaded with 10% FBS-containing medium. After incubation at 37 °C for 24 h, the cells in the upper chamber were removed with cotton swabs. The filters were fixed with 4% paraformaldehyde for 10 min and stained with 1% crystal violet for 15 min. Images were captured by inverted optical microscopy.

Cytoskeleton F-actin filaments Assay

Huh7 or SMMC7721 cells were plated on sterile cover slips and transfected with HA-SQLE or siSQLE as well as their control. After 36 h, cells were fixed with ice-cold 4% paraformaldehyde for 15-30 min and then permeabilized with 0.1% Triton X-100 in PBS for 10 min. After adding 200 μl/well (24-well plate) phalloidin (green fluorescence) staining solution into cells and staining for 0.5 h at room temperature, the samples were observed using a fluorescence microscope with an anti-fluorescence quencher.

RNA-sequencing and analysis

For RNA sequencing, total RNA was extracted using SparkZol Reagent at a concentration of 300˜500 μg/μl according to the manufacturer's protocol. Shanghai Major-bio Biopharm Biotechnology (Shanghai, China) performed the transcriptome sequencing and analyses, and each group had three biological replicates. The data were analyzed on the Majorbio Cloud Platform (*www.majorbio.com*). Differentially expressed genes (DEGs) with $P < 0.05$ were identified.

Dual-Luciferase reporter assay

SMMC7721 cells at a density of 1.2×10^4 cells/well were plated in a 24-well plate 24 h before transfection. pRL-TK (0.08 μg) and the indicated constructs (0.8 μg) were incubated for 36 h using Hieff TransTM Liposomal Transfection Reagent according to the manufacturer's instructions, and pGL3-Basic was used as the control vector. For luciferase assays, SMMC7721 cells were lysed in lysis buffer and oscillated violently on a vortex for 15 min, and promoter activities were detected using a Dual Luciferase Reporter Assay System (Promega, Madison, WI, USA). The data are shown as the ratio of firefly luciferase to renal cell luciferase activity.

Construction of recombinant adenovirus

The recombinant adenoviruses Ad-shSQLE and Ad-shNC were established in our lab using the AdEasy system according to the protocol(Luo *et al.* , 2007). Ad-shSQLE or Ad-shNC was cloned into the shuttle vector pAdTrack-CMV, generating recombinant adenovirus plasmids using BJ5183 cells. Ten˜15 of the smallest colonies were picked and grown in LB medium for 12 h in a 37 orbital shaker. The next day, the plasmid DNA was extracted by the alkaline cleavage method and identified by PacI digestion. To generate

recombinant adenoviruses in HEK-293A packaging cells and prepare viral lysates, a large-scale preparation of high-titer viruses needed at least four rounds of amplification.

Establishment of the mouse tumor model and hematoxylin-eosin staining

SPF male C57BL/6 mice weighing between 18 and 22 g were purchased from the Model Animal Research Center of Nanjing University (Nanjing, China). H22 cells were injected into the abdominal cavity of C57BL/6 mice. After 5˜7 days of injection, the ascites were extracted from tumor-bearing mice and diluted to a concentration of 2×10^7 cells/ml with normal saline. A volume of 200 μl of the cell suspension was subcutaneously injected into the right side of the axillary or liver of each mouse to establish a subcutaneous or orthotopic tumor-bearing mouse model. Orthotopic tumor-bearing mice were randomly divided into two groups and injected with recombinant adenovirus expressing control or shSQLE via the tail vein, and the mice continued to be fed for 12 days. In a subcutaneous tumor-bearing mouse model, when the average tumor size reached 100 mm^3, the mice were divided into two groups: the 0.5% carboxy methyl cellulose (CMC) group and the NB-598 (an inhibitor of SQLE, 10 mg/kg/d) group with gastric infusion 8 times. The tumor sizes were measured once every two days. At the end of the experiment, the serum AFP content in each mouse was measured. The mice were sacrificed, and tumors from either subcutaneous or orthotopic models were collected for further analysis. H&E staining of tumor tissue was performed as previously described (Feldman *et al.* , 2014). The images were captured with optical microscope.

Statistical analysis

Data are expressed as the means ± SEM for triplicates. GraphPad Prism 5 was used for plotting and analysis. Comparisons between two groups were analyzed using the two-tailed Student's t test. p <0.05 was considered statistically significant.

Results

SQLE is specifically upregulated in HCC and predicts poor prognosis

To explore the differentially expressed genes in HCC, RNA-seq analysis was performed on three pairs of clinical HCC and paracancerous tissue samples, and GO, KEGG, and GSEA analyses were conducted by the Majorbio Cloud Platform (*www.majorbio.com*). As shown in Fig. 1A, a total of 30692 genes, including 30153 known and 539 new genes, were detected in the analysis. In addition, a total of 134,592 expressed transcripts, including 122,031 known and 12,561 new transcripts, were identified. Based on the quantitative results of gene expression, the differentially expressed genes (DEGs) between cancer and paracancerous tissues were obtained by using variance analysis software DESeq2, and the threshold value was set as follows: $|\ \log2FC\ | >= 3$ & p value < 0.01. A total of 562 DEGs were identified, among which 210 genes were upregulated and 352 genes were downregulated (Supplementary Table S2). The upregulated genes in HCC include TRIM55, GPC3, AFP, SQLE, TOP2A and so on. Following GO enrichment analysis, HCC-related DEGs were significantly enriched in the spindle checkpoint, DNA replication checkpoint, and chromosome segregation (Fig. 1B). The KEGG enrichment analysis showed that HCC-related DEGs were highly relevant to DNA replication, the cell cycle, oocyte meiosis, mismatch repair, and nitrogen metabolism (Fig. 1C). GSEA further indicated that these DEGs were closely related to the NF-kappa B, HIF-1, cAMP, JAK-STAT, AMPK, and TGF-β signaling pathways (Fig. 1D). AFP is a reliable biomarker in HCC development. Here, the volcano plot revealed that SQLE, an enzyme involved in cholesterol synthesis, was significantly upregulated in HCC in parallel with AFP (Fig. 1E). Furthermore, the TIMER (tumor immune estimation resource) database indicated that SQLE expression was significantly elevated in many tumors, including HCC (Fig. 1F). Similar results were also obtained by using GEPIA (Fig. 1G) and Oncomine (Fig. 1H) database analysis.

To further confirm the above results, we collected clinical samples and examined the expression of SQLE in the tumor and adjacent nontumor tissues. Consistent with the database analysis presented above, both the protein and mRNA expression levels of SQLE in HCC tumor tissues were significantly upregulated compared to those in adjacent nontumor tissues. PCNA expression serves as a control (Fig. 2A&2B). The IHC staining analysis revealed that cytoplasmic SQLE was heavily expressed in HCC tumor tissue compared

to the relatively weak expression in the nontumor tissues (Fig. 2C). Consistent results were also obtained by using tissue microarray analysis (Fig. 2D). To examine the clinical relevance of SQLE, Kaplan–Meier survival analysis revealed that HCC patients with higher SQLE expression had relatively poorer overall survival (OS), recurrence-free survival (RFS), progression-free survival (PFS), and disease-specific survival (DSS) than those with lower SQLE expression (log-rank test, p value was 0.043, 0.081, 0.019, and 0.038, respectively, Fig. 2E). Taken together, SQLE is an important protein in HCC development.

SQLE expression is necessary for HCC cell growth

To understand the role of SQLE in HCC, we first examined SQLE expression in several liver cell lines. As shown in Figure 3A and B, when compared with human normal hepatocytes (LO2 cells), both the gene and protein expression levels of SQLE were significantly upregulated in an array of HCC cell lines, including Huh7, SMMC7721, HepG2.2.15, and PLC/PRF/5 cells. Huh7 and SMMC7721 cells were selected for the transient knockdown of SQLE expression with siRNAs (Fig. 3C). The MTT assay indicated a considerable decrease in cell viability after SQLE knockdown compared to the control group (Fig. 3D), and silencing of SQLE expression also significantly suppressed clone formation in both SMMC7721 and Huh7 cells (Fig. 3E). The Annexin V-FITC/PI apoptosis assay revealed that the reduced SQLE expression in either SMMC7721 or Huh7 cells triggered significant apoptosis occurrence (Fig. 3F). Similarly, the EdU incorporation assay showed that Huh7 and SMMC7721 cell proliferation was significantly suppressed in cells after SQLE expression was silenced (Fig. 3G). In contrast, when SQLE was overexpressed in Huh7 and SMMC7721 cells (Fig. 3H), cell growth was significantly promoted compared with that of control cells (Fig. 3I). Similar results were also observed in colony formation experiments (Fig. 3J) and EdU incorporation assays (Fig. 3K).

SQLE promotes HCC cell migration and F-actin assembly

To examine whether SQLE influences cell migration in HCC, we first observed a noticeable reduction in wound healing in both Huh7 and SMMC7721 cells after endogenous SQLE expression was silenced (Fig. 4A); in contrast, SQLE overexpression significantly accelerated wound healing (Fig. 4B). The numbers of migrating Huh7 and SMMC7721 cells were significantly reduced when SQLE expression was silenced in the Transwell migration assay (Fig. 4C). SQLE overexpression, however, increased the cell migration capacity (Fig. 4D). Epithelial-mesenchymal transformation (EMT) is a remarkable hallmark of tumor cell metastasis. In this study, we observed that the silencing of SQLE led to a significant reduction in N-cadherin and vimentin expression but an increase in E-cadherin expression at both the protein and mRNA levels. SQLE overexpression, however, had the opposite result (Fig. 4E-H). In addition, SQLE knockdown markedly prevented F-actin formation (Fig. 4I), while forced SQLE expression facilitated the overgrowth of F-actin (Fig. 4J) in Huh7 and SMMC2771 cells, suggesting a pivotal role of SQLE in cytoskeletal reorganization that facilitates tumor cell migration.

ΣΧΛΕ ινςρεασες Η$^{\text{ϝϝ}}$ ςελλ ινασιενεσς βψ αςτιατινγ τηεΤΓΦ-β/ΣΜΑΔ σιγναλινγ πατηωαψ

To explore the underlying mechanism of SQLE in HCC, unbiased transcriptome analysis was performed by RNA sequencing (RNA-seq) on samples of Huh7 cells with or without SQLE silencing, and the data were analyzed by the Majorbio Cloud Platform. As a result, a total of 31270 genes, including 30912 known genes and 358 yet-unidentified genes, were detected. There were 143,463 expressed transcripts, including 130,298 known transcripts and 13,165 new transcripts. The differentially expressed genes were analyzed by DESeq2 software. The results demonstrated that there were a total of 800 differentially expressed genes (NC *vs* siSQLE), of which 294 were upregulated and 506 were downregulated genes (Fig. 5A and Supplementary Table S3). The KEGG enrichment analysis showed that TGF-β signaling is the most relevant biological pathway to SQLE's function in Huh7 cells (Fig. 5B). On the other hand, the GO enrichment analysis revealed that SQLE is closely related to cell contraction, movement, wound healing, and matrix reorganization, all of which are typical characteristics of tumor cell migration (Fig. 5C).

TGF-β/SMAD signaling is critical in HCC development(Li *et al.* , 2020). In principle, the TGF-β super-

family comprises nearly 30 kinds of structurally and functionally related proteins, such as BMPs, GDFs, GDNFs, and activins(Massague *et al.* , 2000). TGF-β exerts its biological activity through SMAD-dependent or SMAD-independent pathways(Zhang, 2017). TGF-β binds to and activates its receptors and then activates downstream SMAD proteins through phosphorylation and nuclear translocation(Li *et al.* , 2020). To understand the relationship between SQLE and TGF-β/SMAD signaling in HCC, we first examined the TGF-β1, TGF-β2, and TGF-β3 mRNA levels in Huh7 and SMMC7721 cells after transfection with siRNAs against SQLE. Among them, TGF-β1 mRNA was significantly decreased by SQLE knockdown in cells (Fig. 5D). Silencing of SQLE also decreased TGF-β1 protein expression, and further, the phosphorylation of SMAD2 and SMAD3 in both Huh7 and SMMC7721 cells (Fig. 5E) and SQLE overexpression, however, had the opposite result (Fig. 5F). Functional experiments revealed that TGF-β-induced wound healing was significantly retarded in cells after transfection with siRNAs against SQLE (Fig. 5G). Similar results were also obtained in the Transwell migration assay (Fig. 5H). TGF-β1 is a well-established regulator of the process of EMT(Moustakas*et al.* , 2007). Here, TGF-β-induced EMT, characterized by mRNA increases in N-cadherin, vimentin, snail, Zeb-1, and Zeb-2, a decrease in E-cadherin mRNA, and an increase in TGF-β, SMAD2 and SMAD3 phosphorylation, was largely alleviated in cells after transfection with SQLE siRNAs (Fig. 5I-J). SB-431542 is an inhibitor of TGF-β receptor-mediated signaling. SQLE-induced EMT (Fig. 5K) and increases in cell wound-healing activity and migration were largely attenuated in the presence of SB-431542 (Fig. 5L-M). A previous study revealed that SQLE can promote HCC development by stimulating DNMT3A expression and its activity, leading to activation of the PTEN/PI3K/AKT/mTOR signaling cascade. We found that the upregulation of DNMT3A and PTEN mRNA levels was largely suppressed by SB-431542 (Fig. 5N); as such, SQLE also regulates PTEN and DNMT3A expression through TGF-β/SMAD signaling.

The protumorigenic activity of SQLE in tumor-bearing models of hepatocellular carcinoma

To further characterize the protumorigenic role of SQLE in vivo, we constructed an orthotopic transplantation tumor model of HCC in mice bearing intraperitoneal H22 tumors. Adenovirus-mediated SQLE shRNA constructs were injected into mice via the tail vein. After experiments, mouse livers were removed, and immunoblotting analysis revealed that the*in vivo* delivery of Ad-shSQLE led to a significant reduction in SQLE protein expression in mouse livers (Fig. 6A). Under this condition, the average H22 tumor size in mice challenged with shSQLE was significantly smaller than that in control mice (Fig. 6B). In fact, the liver weight of mice injected with shSQLE was also lighter than that of control mice (Fig. 6C). Furthermore, SQLE silencing in mice correlated with a reduction in serum AFP content (Fig. 6D). H&E staining of liver tissue further confirmed the reduced tumor lesions in mice challenged with shSQLE (Fig. 6E). Notably, lung metastasis nodules were found in either control or shSQLE-injected mice, but there were significantly fewer nodules in SQLE-silenced mice than in control mice (Fig. 6F). The decreased SQLE expression in tumor tissue by siRNA silencing also correlated with a reduction in PCNA expression and the phosphorylation of SMAD2 or SMAD3, as confirmed by IHC staining (Fig. 6G-H).

STRAP is critically involved in the oncogenic effect of SQLE on HCC development

To understand why SQLE is able to promote HCC development, we noted that among the downregulated genes in Huh7 cells after transfection with SQLE siRNA, STRAP was one of the genes that was strongly inhibited by SQLE. To verify the RNA-seq analysis, silencing of SQLE expression in SMMC7721 cells significantly decreased STRAP mRNA expression, but SQLE overexpression increased it (Fig. 7A). A previous study indicated that STRAP is an oncogene (Wang *et al.* , 2019). Here, TCGA analysis revealed that STRAP was highly expressed in HCC tumor tissue compared with adjacent nontumor tissues (Fig. 7B), and further, a high expression level of STRAP in HCC patients correlated with poor prognosis (Fig. 7C). Notably, GEPIA2 analysis revealed that SQLE was positively correlated with STRAP expression in HCC patients but not in nontumor tissue (Fig. 7D). Taken together, we speculated that STRAP could be an important gene involved in the protumorigenic effect of SQLE on HCC.

To test the above speculation, STRAP expression in Huh7 and SMMC7721 cells was silenced by siRNAs (Fig. 7E). As a result, SQLE overexpression-induced Huh7 and SMMC7721 cell growth was largely attenuated

in cells after STRAP siRNA transfection (Fig. 7F). Furthermore, the enhanced F-actin expression (Fig. 7G), tumor cell proliferation (Fig. 7H), migration (Fig. 7I), and wound-healing ability (Fig. 7J) in SQLE-overexpressing cells were all largely attenuated by STRAP silencing. Surprisingly, SQLE-induced SMAD2/3 phosphorylation and TGFβ1 expression were almost completely suppressed in HCC cells after transfection with STRAP siRNAs, suggesting that SQLE-activated TGFβ1/SMAD signaling is critically dependent on STRAP (Fig. 7K).

ΣΧΛΕ προμοτες ΣΤΡΑΠ γενε τρανσϛριπτιον τηρουγη ΑΠ-2α

To clarify why STRAP gene expression is regulated by SQLE, we first used actinomycin D (Act D) to halt global gene transcription and then observed whether SQLE affects the mRNA stability of STRAP. As a result, the half-life of STRAP mRNA was not affected in SQLE-silenced cells compared with control cells (Fig. 8A), suggesting that SQLE may increase STRAP expression at the transcriptional level.

To confirm the effects of SQLE on STRAP gene transcription and the potential cis-elements involved in this process, we constructed the full-length human STRAP promoter (-1029 to +213) and its truncated variants (-738 to +213, -498 to +213, -148 to +213, -28 to +213 bp). The results revealed that SQLE was able to increase the promoter activity of STRAP, and cis-elements between -148 and -28 are essential for the activation effects of SQLE (Fig. 8B). Bioinformatics analysis (JASPAR databases) indicated that SP1, c/EBPβ, and AP-2α are putative transcription factors that can bind with cis-elements within the -148 to -28 region (Fig. 8C upper). To identify the potential transcription factors, we continued to construct plasmids with mutations in SP1, c/EBP β, and AP-2α potential binding sites (Fig. 8C below). The results of the luciferase reporter assay showed that the effects of SQLE on STRAP promoter transactivation were largely attenuated when the AP-2α binding site but not the SP1 or c/EBP β binding site was mutated (Fig. 8D). To further verify the results, SP1, c/EBP β or AP-2α expression was silenced in SMMC7721 cells. As shown in Fig. 8E-F, SQLE-induced STRAP promoter activation was significantly alleviated in cells with reduced expression of AP-2α but not SP1 or c/EBP β. Accordingly, SQLE overexpression-induced STRAP gene expression was also largely suppressed in cells transfected with AP-2α siRNA but not SP1 or c/EBP β (Fig. 8G). As such, AP-2α is the key trans-acting factor involved in STRAP gene expression by SQLE.

The SQLE inhibitor NB-598 suppresses HCC growth both in vitro and in vivo.

In view of the important role of SQLE in HCC development, we tried to examine whether SQLE could be a target for HCC therapy. NB-598, as a terbinafine derivative obtained by modification of aromatic groups, is a specific inhibitor of SQLE that can suppress triglyceride biosynthesis (Hiyoshi *et al.* , 2003) and effectively decrease serum cholesterol and triacylglycerol levels (Horie *et al.* , 1993). We set out to determine whether NB-598 can be repurposed for the prevention or treatment of HCC. Huh7 and SMMC7721 cells were treated with different doses of NB-598 (0.005, 0.01, 0.05, 0.1, 0.5, 1, 2 μM), and the results demonstrated that NB-598 significantly inhibited the viability of Huh7 and SMMC7721 cells, as examined by MTT (Fig. 9A) and colony formation assays (Fig. 9B). Consistently, NB-598 reduced EdU incorporation in Huh7 and SMMC7721 cells (Fig. 9C) and dose-dependently triggered cell apoptosis, as examined by Annexin V-FITC/PI staining assay (Fig. 9D). The western blot assay indicated that NB-598 inhibited TGF-β1 expression and SMAD2/3 phosphorylation in a dose-dependent manner, in line with the results obtained by SQLE knockdown (Fig. 9E). In addition to the*in vitro* results, the *in vivo* experiments revealed that NB-598 significantly suppressed the tumor growth of subcutaneous H22 xenografts (Fig.). Furthermore, the H22 tumor weight in NB-598-treated mice was significantly lighter than that in control mice (Fig. 9G-I). NB-598 treatment also decreased the serum content of AFP in tumor-bearing mice (Fig. 9J). H&E staining of tumor tissues further confirmed the antitumorigenesis effect of NB-598 (Fig. 9K). Moreover, NB-598 inhibited SQLE and PCNA protein expression in tumor tissue (Fig. 9L, M).

Discussion

In the present study, both bioinformatics and RNA sequencing analysis of HCC samples revealed that SQLE is highly expressed in HCC and is associated with poor prognosis. Mechanistically, SQLE promoted HCC development by activating the TGF-β/SMAD signaling pathway, and STRAP is an essential protein involved

in this process. To potentiate the activation of TGF-β/SMAD signaling, SQLE even increased STRAP transcription by promoting AP-2α binding with the promoter. A chemical inhibitor of SQLE significantly suppressed HCC development both *in vitro* and *in vivo* . All of these data suggest that SQLE is a promising target for HCC therapy and drug development.

Increasing evidence reveals that TGF-β signaling is a promising target in cancer therapy (Colak *et al.* , 2017). In the late stage of cancer development, the activation of TGF-β signaling promotes tumorigenesis, metastasis, and chemoresistance (Hu *et al.* , 2018). Among TGF-β isoforms, TGF-β1 plays a crucial role in EMT and fibrogenesis by activating SMAD2/3 phosphorylation (Chen*et al.* , 2018). In this study, we identified a novel role of SQLE in regulating TGF-β signaling in HCC. The evidence is as follows: SQLE promoted N-cadherin, vimentin, snail, zeb1, and zeb2 expression but decreased E-cadherin expression. SQLE overexpression increased cytoskeletal rearrangement by enhancing F-actin expression. TGF-β1 mRNA, but not TGF-β2 or TGF-β3 mRNA expression, was upregulated by SQLE; as a result, SQLE triggered SMAD2/3 phosphorylation. All of these events could be reversed by SQLE knockdown in HCC. In parallel, SB431542, a selective inhibitor of TGF-β signaling, abrogated the promoting effect of SQLE on EMT, cell migration, and cytoskeletal rearrangement. Thus, by activating TGF-β signaling, SQLE plays a key role in liver cancer metastasis. A previous study indicated that SQLE has a pro-oncogenic role in NAFLD-induced HCC by activating the PTEN/PI3K/AKT/mTOR signaling cascade(Liu *et al.* , 2018). More specifically, SQLE mediated oxidative stress and then stimulated DNMT3A expression and activity, decreasing PTEN via promoter methylation. In fact, the SMAD3-PTEN signaling axis determines the cellular responses to TGF-β signaling in a previous study(Eritja*et al.* , 2017), and DNMTs are regulated by TGF-β signaling. The stimulatory effects of TGF-β on SOCS3 promoter methylation were mediated by DNMT3A in a SMAD-dependent manner (Dees*et al.* , 2020). Consistent with these results, we found here that the regulation of PTEN and DNMT3A by SQLE was abolished in the presence of SB-431542. Taken together, in addition to serving as an enzyme in cholesterol synthesis, SQLE emerges as an important molecule involved in TGF-β signaling that acts upstream of DNMT3A, PTEN, and PI3K activation.

To understand the mechanism of TGF-β signaling activation by SQLE, we identified that STRAP is critically involved by RNA-seq analysis and many other functional experiments. As a serine-threonine kinase receptor-associated protein, STRAP has been reported as an oncogene in a variety of cancers, including neuroblastoma(Bownes *et al.* , 2021) and pancreatic ductal adenocarcinoma(Hu*et al.* , 2020). STRAP is one of the WD40 superfamily proteins that contains seven WD40-repeat domains. In general, proteins containing WD domains have regulatory effects on signal transduction, gene transcription, RNA synthesis and processing, programmed cell death, and vesicle transport (Datta *et al.* , 1998; Halder *et al.* , 2006). A previous study revealed that STRAP can activate the Wnt/β-catenin signaling (Wang *et al.* , 2019) and MEK/ERK signaling (Halder *et al.* , 2006) pathways to stimulate cell growth. Notably, the regulatory effect of STRAP on TGF-β signaling is rather complicated, mainly because TGF-β signaling has dual roles in regulating cancer cell destiny (Syed, 2016). STRAP was found to negatively regulate the TGF-β/SMAD7 signaling pathway by stabilizing the binding between the TGF-β receptor and SMAD7 (Halder *et al.* , 2006); it can also prevent the binding between SMAD2/3 and the TGF-β receptor, thus inhibiting TGF-β signaling(Datta *et al.* , 1998). However, another study also found that STRAP inhibited classic TGF-β/SMAD7 but promoted TGF-β/SMAD3 signaling, and as a result, STRAP overexpression alleviated TGF-β-induced cell growth inhibition and induced tumorigenicity in lung adenocarcinoma and colonic adenocarcinoma(Halder *et al.* , 2006). In our study, we found that SQLE upregulated the mRNA expression of STRAP, and there was a positive correlation between SQLE and STRAP in HCC. Further experiments demonstrated that SQLE enhanced STRAP transcription through AP-2α. Meanwhile, the effect of SQLE on the TGF-β/SMAD signaling pathway was dependent on STRAP. From the above results, we presume that in SQLE-mediated activation of TGF-β signaling and HCC development, STRAP was actively upregulated by SQLE to probably influence the protein interactions between SMADs and TGF-β receptors, which favors SMAD2/3 phosphorylation. The specific mechanism remains to be further explored.

The SQLE amino acid sequence homology in mammals is approximately 83-93%, and in fungi, it is 65-95%; however, the sequence homology of SQLE between fungi and mammals is only 30.2-35%, which provides a

theoretical basis for the specificity and broad spectrum of antifungal drugs(Lee *et al.* , 2004; Motavaze *et al.* , 2008). At present, the main reported SQLE inhibitors are antifungal compounds, including terbinafine and naphthalene, and lipid-lowering compounds, including propionamide, natural compounds, and their derivatives(Sanossian *et al.* , 2008; Nowosielski *et al.* , 2011). Based on the chemical modification of terbinafine, a series of compounds that exert inhibitory effects on mammalian SQLE were produced, and the NB-598 compound was obtained by modification of the aromatic group of terbinafine in 1990. NB-598 is a strong competitive inhibitor of mammalian SQLE, effectively inhibiting rat and dog liver microsome SQLE enzymes (Horie *et al.* , 1990; Hidaka *et al.* , 1991). In the present study, NB-598 effectively inhibited the proliferation of Huh7 and SMMC7721 cells, induced cell apoptosis by inhibiting the TGF-β/Smad signaling pathway, and suppressed the occurrence of HCC in *vivo* . These results suggest that NB-598 has the potential to be repurposed for HCC treatment.

Data Availability

All data supporting the paper are presented in the paper and/or the Supplementary Materials. The original datasets are also available from the corresponding author upon request.

Acknowledgments

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Author contributions

Z.R.Z., J.C., F.Z.Y. and W.Y. designed the overall study. W.W., H.J. and X.J.J. analyzed the data, Z.R. Z and W.Y. wrote the paper, W.W., H.J. and Y.Z. C conducted the clinical experiment, Z.R. Z conducted the experiments. All authors have read and approved the article.

Conflicts of interest

The authors declare that they have no conflicts of interest.

Declaration of transparency and scientific rigour

This Declaration acknowledges that this paper adheres to the principles for transparent reporting and scientific rigour of preclinical research as stated in the BJP guidelines for Design and Analysis, Immunoblotting and Immunochemistry, and Animal Experimentation, and as recommended by funding agencies, publishers and other organizations engaged with supporting research.

Figure Legends

Fig 1 . RNA-Seq analysis of differentially expressed genes (DEGs) inclinical HCC and paracancerous tissues. (A) Heatmap showing 562 genes differentially expressed in clinical HCC and paracancerous tissues (T: clinical HCC tissue; NT: clinical HCC adjacent tissue). (B-D) GO, KEGG and GSEA enrichment analysis of DEGs. (E) Volcano plot of DEGs in NT vs. T. Upregulated genes are shown in red circles, downregulated genes are shown in green circles, and genes with no significance are shown in gray circles. (F) SQLE expression in multiple cancers in the TIMER database (*https://cistrome.shinyapps.io/timer/*). (G) The mRNA level of SQLE expression among adjacent nontumor and HCC tissues in the GEPIA2 database (*http://gepia2.cancer-pku.cn/#index*). (H) The mRNA level of SQLE expression among adjacent nontumor and HCC tissues in the Oncomine database.$^{*}P$ <0.05,$^{***}P$ <0.001.

Fig 2 .SQLEis upregulated in HCC tumor tissues with unfavorable prognosis.(A) Western blotting analyses of the SQLE and PCNA protein expression levels in six pairs of clinical HCC and adjacent nontumor tissue samples; GAPDH was used as the internal loading control. T, tumor tissue; NT, nontumor tissue.

(B) RT–PCR analyses of SQLE in six pairs of clinical HCC and adjacent nontumor tissue samples. (C) Representative images of IHC staining for SQLE in clinical HCC and adjacent nontumor tissue samples. Magnification: 100× and 50×. (D) Representative images of IHC staining for SQLE in a clinical sample tissue microarray. Magnification: 200× and 20×. (E) The Kaplan–Meier plot for the survival of HCC patients according to SQLE expression levels (*http://kmplot.com/analysis/*).

Fig 3 . SQLE expression is necessary for the survival of HCC cells. (A, B) The protein and mRNA levels of SQLE expression in several HCC cell lines detected by western blot and RT–PCR. (C) Western blot analysis of SQLE expression in Huh7 and SMMC7721 cells transfected with siRNAs against SQLE (siSQLE). (D) MTT assay detected the viability of HCC cells with or without SQLE knockdown. (E) The effect of SQLE on clone formation in Huh7 and SMMC7721 cells. (F) The cells transfected with siNC or siSQLE were analyzed using an Annexin V-FITC/PI assay. (G) Representative images of the EdU experiment of lower SQLE expression levels. Scale bars: 200 μm. (H) Western blot analysis confirming the effect of HA-SQLE on overexpressing SQLE. (I) The cell viability in cells with or without SQLE overexpression was detected by MTT assay. (J) The effect of SQLE overexpression on clone formation. (K) Representative images of the EdU experiment of higher SQLE expression levels and coincident with that of FCM. Scale bars: 200 μm.

Fig 4 .SQLE is positively correlated with the migration of HCC cells.(A-D) Wound healing assays and migration assays were conducted to determine the effect of SQLE on the migratory abilities of Huh7 and SMMC7721 cells. (E-H) Western blot and qRT–PCR showed the changes in EMT markers after SQLE knockdown or overexpression. (I-J) Huh7 and SMMC7721 cells were transfected with siNC and siSQLE or pcDNA3.1-HA and HA-SQLE plasmids, followed by immunofluorescence assay to observe F-actin (green). Scale bars: 200 μm.

Fig 5 . ΣΧΛΕ προμοτες μεταστασις βψ αςτιατινγ τηε ΤΓΦ-β/ΣΜΑΔ σιγναλινγ πατηωαψ ιν Η"". (A) Heatmap shows differentially expressed genes in Huh7 cells with or without SQLE knockdown. (B, C) KEGG and GO enrichment analysis of DEGs in Huh7 cells (NC *vs* . siSQLE#2). (D) qRT–PCR analysis of TGFβ1, TGFβ2, and TGFβ3 in SQLE-knockdown Huh7 and SMMC7721 cells. GAPDH was used as a control, and data are expressed as the mean ± SEM (n = 3). (E, F) The protein expression of SMAD2/3 and p-SMAD2/3 in Huh7 and SMMC7721 cells transfected with siSQLE#2 or HA-SQLE plasmid was determined by western blot analysis. Tubulin was used as the loading control. (G, H) Huh7 and SMMC7721 cells were treated with siSQLE#2 for 36 h in the presence of TGF-β1 (5 ng/ml) for 12 h. Wound healing assays and migration assays were used to evaluate cell metastasis viability. (I) qRT–PCR showed the changes in EMT markers after treatment with TGF-β1 and siSQLE#2.$^{**}P < 0.01$ compared with the control group; $^{\#\#}P < 0.01$ compared with the TGF-β1-treated group. (J) SQLE-siRNA reversed the upregulation effect of TGF-β1 on TGF/SMAD signaling pathway proteins. Tubulin was used as the loading control. (K-M) Huh7 and SMMC7721 cells were treated with HA-SQLE for 36 h with or without SB-431542 (5 μM) for 24 h. qRT–PCR (K) showed the changes in EMT markers, and wound healing assays (L) and migration assays (M) evaluated cell metastasis viability. (N) qRT–PCR showed the changes in the PTEN and DNMT3A genes after SB-431542 treatment. $^{*}P< 0.05$, $^{**}P < 0.01$ compared with HA-SQLE group.

Fig 6 . Knockdown of SQLE inhibits tumor formation in C57BL/6 mice. (A) Immunoblotting of SQLE expression in liver tissues from shNC and shSQLE mice. (B) Gross morphological images of livers from orthotopic transplantation tumor models in C57BL/6 mice (n=6). The red circles are nodules of tumor. (C) Liver weights of shNC and shSQLE groups. (D) The AFP content in the orbital blood of mice injected with shNC or shSQLE adenovirus. (E-F) Representative images of H&E staining of liver and lung tissues in the shNC and shSQLE groups. Scale bar, 2000 μm and 200 μm. (G-H) Representative IHC staining images of SQLE, PCNA, SMAD2 and SMAD3 expression in liver tissues in the shNC and shSQLE groups. Scale bar, 2000 μm and 200 μm. $^{*}P <0.05$, $^{***}P <0.001$.

Fig 7 . STRAP is critically involved in the oncogenic effect of SQLE on HCC development. (A) SMMC7721 cells were transfected with siSQLE#2 or HA-SQLE, and the mRNA levels of STRAP were determined by RT–PCR. (B) STRAP expression levels in HCC (n=369) and nontumor tissues (n=160) in

the GEPIA2 database. (C) The overall survival curves of HCC patients with high (n=111) and low (n=253) expression of STRAP using Kaplan–Meier Plotter. (D) Correlations among SQLE and STRAP in HCC tumor and nontumor tissues were denoted with Kendall, Spearman and Pearson's correlation coefficients. (E) Western blot verified the interference effect of siSTRAP. (F) The viability of Huh7 and SMMC7721 cells overexpressing SQLE with or without transferred siSTRAP was detected by MTT assay (***P <0.001). (G) Immunofluorescence assay displayed F-actin (green) formation in HCC cells cotransfected with HA-SQLE and siSTRAP. Scale bars: 200 μm. (H) The effect of siSTRAP overexpression on clone formation in Huh7 and SMMC7721 cells. (I-J) Migration assays and wound-healing assays were performed to observe the migratory abilities of HCC cells cotransfected with HA-SQLE and siSTRAP. (K) Knockdown of STRAP reversed the upregulation effect of HA-SQLE on TGF-β/SMAD signaling pathway proteins. Tubulin was used as the loading control.

Fig 8 . **ΣΧΛΕ προμοτες ΣΤΡΑΠ γενε τρανσςριπτιον τηρουγη ΑΠ-2α.** (A) SQLE did not affect the STRAP mRNA half-life in SMMC7721 cells. SMMC7721 cells were transfected with siNC or siSQLE#2, incubated for 24 hours and treated with ActD (4 μg/ml) for 2, 4 and 6 h. Gene expressions were measured with RT–PCR. (B) Serial deletion mutant analysis of the STRAP promoter. SMMC7721 cells were transfected with the indicated plasmids (0.8 μg) and pRL plasmid (0.08 μg) and incubated with or without siSQLE#2/HA-SQLE for 36 h. The transcriptional activity of truncated STRAP was measured according to the protocols. (C) The transcription factor binding sites of the human STRAP gene were predicted by PROMO HOME PAGE (upper), and variants of the STRAP promoter with point mutations in the indicated sites were constructed (below). (D) AP-2α binding sites played an important role during the transcriptional process. STRAP promoter constructs with mutations in specific SP1, C/EBP beta and AP-2α sites were transfected into SMMC7721 cells incubated with siSQLE#2/HA-SQLE or vector, and luciferase activity was detected according to the protocols. (E) Effects of SP1, C/EBP beta and AP-2α siRNA on STRAP luciferase reporting activity in SMMC7721 cells. (F) The interference efficiency of SP1, C/EBP beta and AP-2α siRNA in SMMC7721 cells. (G) Effects of SP1, C/EBP beta and AP-2α siRNA on STRAP mRNA expression in SMMC7721 cells incubated with HA-SQLE. Data are shown as means ± SEM. *P< 0.05, **P < 0.01, ***P < 0.001, ns indicates nonsignificant.

Fig 9 . The **SQLE inhibitor NB-598 suppresses HCC growth in vitro and in vivo.** (A, B) NB-598 treatment inhibited cell viability and colony formation in Huh7 and SMMC7721 cells. (C) Representative images of the EdU experiment of NB-598 treatment in HCC cells. Scale bars: 200 μm. (D) The Annexin V-FITC/PI assay detected that NB-598 induced apoptosis in Huh7 and SMMC7721 cells. (E) Western blotting showed the suppression efficiency of the TGF/SMAD signaling pathway after treatment with NB-598. (F) Representative images of tumor size in C57BL/6 mice injected subcutaneously with H22 cells treated with either CMC or NB-598 (n =5, for each experimental group). (G-I) The mouse weight, tumor growth curves and weight of xenografts in the control and NB-598 groups are shown. The data shown are the mean ± SEM. (J) The content of AFP in the orbital blood of mice treated with CMC or NB-598. The data shown are the mean ± SEM. (K) H&E staining of tumor tissues in the control and NB-598 groups. Scale bar, 2000 μm and 200 μm. (L, M) Representative IHC staining of SQLE and PCNA expression in the control and NB-598 groups is shown. Scale bar, 2000 μm and 200 μm. *P <0.05, **P <0.01, ***P <0.001.

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