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High Squalene Epoxidase in Tumors Predicts Worse Survival in Patients With Hepatocellular Carcinoma: Integrated Bioinformatic Analysis on NAFLD and HCC

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Abstract

This study aimed to identify candidate biomarkers for predicting outcomes in nonalcoholic fatty liver disease (NAFLD) and hepatocellular carcinoma (HCC). Using Gene Expression Omnibus and The Cancer Genome Atlas (TCGA) databases, we identified common upregulated differential expressed genes (DEGs) in patients with NAFLD/nonalcoholic steatohepatitis (NASH) and HCC and conducted survival analysis of these upregulated DEGs with HCC outcomes. Two common upregulated DEGs including squalene epoxidase (SQLE) and EPPK1 messenger RNA (mRNA) were significantly upregulated in NAFLD, NASH, and HCC tissues, both in GSE45436 ($P < .001$) and TCGA profile ($P < .001$). Both SQLE and EPPK1 mRNA were upregulated in 15.56% and 8.06% patients with HCC in TCGA profile. Overexpression of SQLE in tumors was significantly associated with worse overall survival (OS) and disease-free survival (DFS) in patients with HCC (log-rank $P = .027$ and log-rank $P = .048$, respectively), while no statistical significances of OS and DFS were found in EPPK1 groups (both log-rank $P > .05$). For validation, SQLE upregulation contributed to significantly worse OS in patients wih HCC using Kaplan-Meier plotter analysis (hazard ratio = 1.43, 95% confidence interval: 1.01-2.02, log-rank $P = .043$). In addition, high level of SQLE significantly associated with advanced neoplasm histologic grade, advanced AJCC stage, and α-fetoprotein elevation ($P = .036$, .045, and .029, respectively). Squalene epoxidase is associated with OS and DFS and serves as a novel prognostic biomarker for patients with HCC.

Keywords

squalene epoxidase, hepatocellular carcinoma, α-fetoprotein, survival, AJCC stage

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Introduction

Liver cancer, comprising 75% to 85% cases of hepatocellular carcinoma (HCC), is predicted to be the sixth most commonly diagnosed cancer and the fourth leading cancer-related deaths worldwide in 2018, with about 841 000 new cases and 782 000 deaths annually.[1-4] In addition, the incidence of HCC will continue to rise until 2030 based on a the Surveillance, Epidemiology, and End Results (SEER) program registry projects study.[5] Precise estimation of prognosis plays a critical role in treatment decision in patients with HCC. Thus, identifying novel biomarkers for predicting HCC prognosis and revealing target for treatment are urgently required.[6,7]

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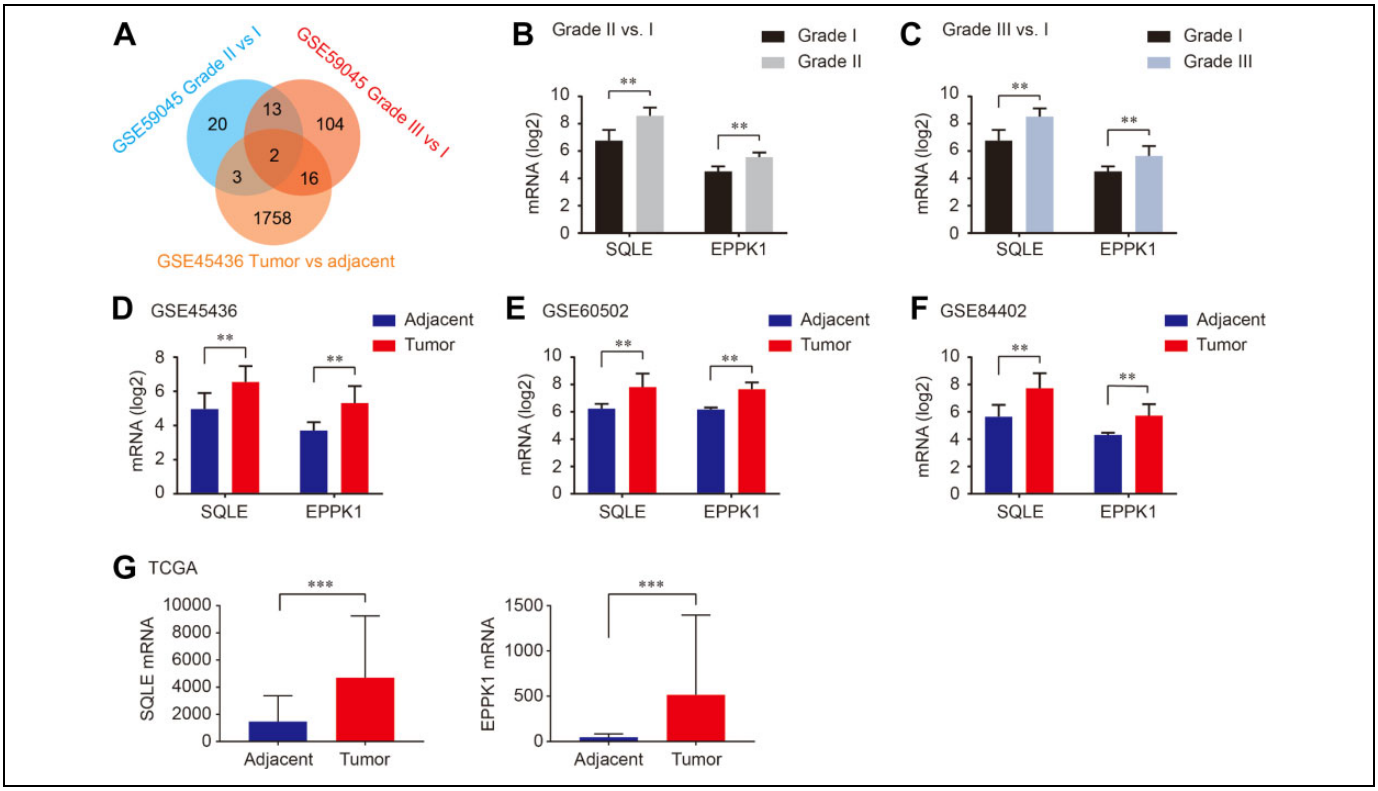

Figure 1. Two commonly upregulated DEGs including SQLE and EPPK1 were identified between GSE59045 and GSE45436 (A), SQLE mRNA and EPPK1 mRNA were overexpressed in steatosis grade II and grade III compared with grade I (B and C), SQLE mRNA and EPPK1 mRNA were upregulated in tumor tissues than those in adjacent tissues in patients with HCC (D-F), which was validated in TCGA database (G). DEGs indicates differential expressed genes; HCC, hepatocellular carcinoma; mRNA, messenger RNA; SQLE, squalene epoxidase; TCGA, The Cancer Genome Atlas.

The rising prevalence of obesity is considered a contributory factor to the observed increasing incidence of HCC.[8] Emerging evidences revealed that paralleling the epidemic of metabolic syndrome and obesity worldwide,[9] nonalcoholic fatty liver disease (NAFLD) and nonalcoholic steatohepatitis (NASH) served as independent risk factors for HCC, even in the absence of cirrhosis.[10-13] The pathogenesis of HCC arising in the context of NAFLD as underlying liver disease has not been well elucidated.[14] The molecular classifications of HCCs did not provide a signature pathognomonic for HCC in NAFLD/NASH.[15] In this study, we identified common upregulated differential expressed genes (DEGs) in patients with NAFLD/NASH and HCC and conducted survival analysis of these upregulated DEGs with HCC outcomes, in hope of providing screening suggestions of NAFLD/NASH and useful insights into the pathogenesis and progression of HCC.

Materials and Methods

Source of Data

GSE59045 and GSE45436 profiles were obtained from Gene Expression Omnibus (GEO, https://www.ncbi.nlm.nih.gov/geo/) database. Patients with NAFLD/NASH in GSE59045 were grouped according to liver tissue histology: 6 patients in group I (<5% steatosis), 4 in group II (NAFLD, 30% to 50% steatosis), and 5 in group III (NASH).[16] GSE45436 were composed with GSE45267, GSE45434 (training sets), and GSE45435 (validation set). Eighteen frozen tumorous and adjacent nontumorous liver tissues were used for gene expression profiling study in GSE60502 and 14 pairs of HCC tissues and corresponding noncancerous tissues were isolated and purified in GSE84402. Microarray analyses were performed to identify genes expressed differentially among groups. Affymetrix Human Gene Expression Array platform was used for microarray analysis in GSE59045, Affymetrix Human Genome U133A Array was used in GSE60502, and Affymetrix Human Genome U133 Plus 2.0 Array platforms were used in GSE45436 and GSE84402.

Screening Commonly Upregulated DEGs

The gene expression data were processed using the Robust Multi-array Average algorithm. To investigate DEGs in transcriptome between groups including normal, NAFLD, and NASH in morbidly obese patients, and tumor and adjacent normal tissues in patients with HCC, Affy, AffyPLM, and Limma packages were used for quality assessment and identifying DEGs in each GEO profile based on the microarray platform. The criteria for selection of DEGs were set as

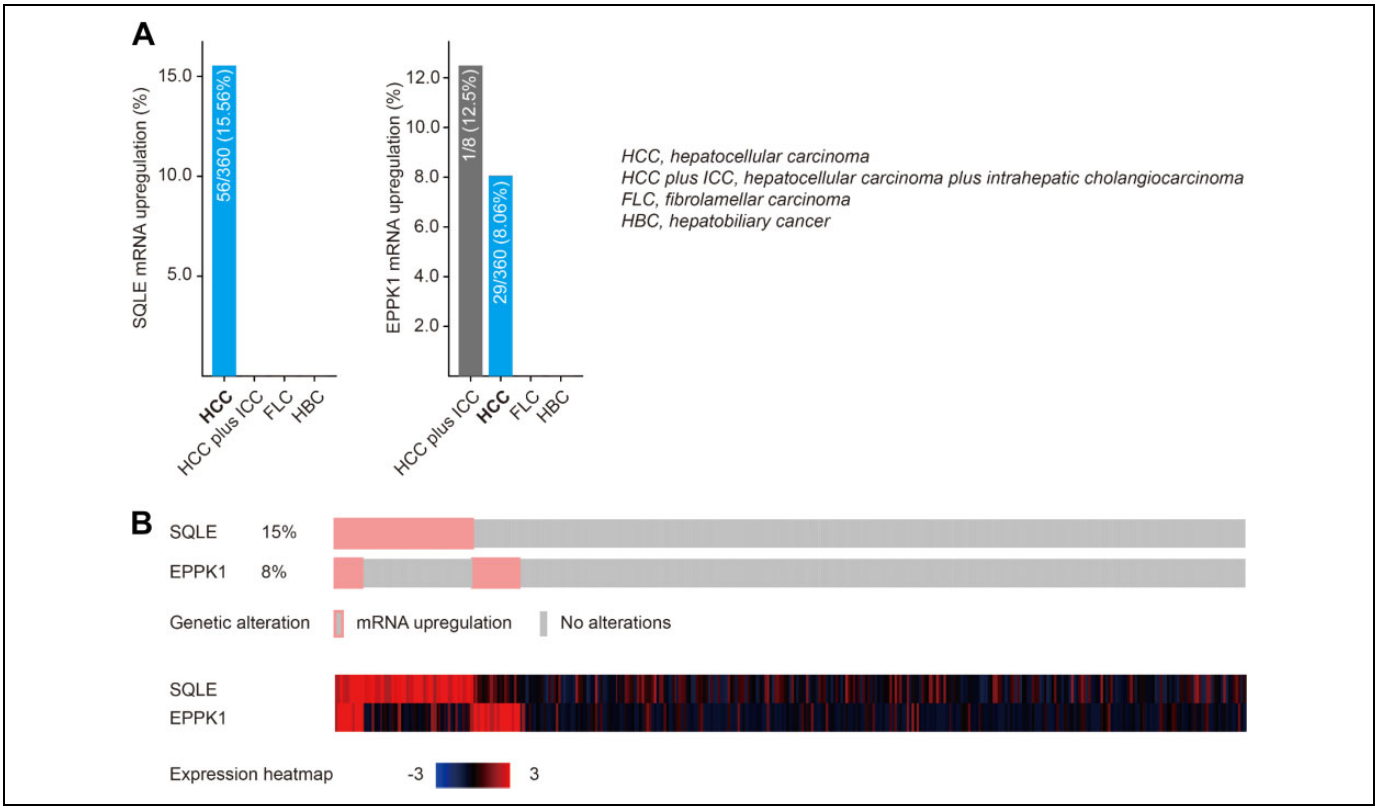

Figure 2. Upregulation frequency of SQLE and EPPK1 mRNA in different liver cancer types using cBioPortal online analysis. mRNA indicates messenger RNA; SQLE, squalene epoxidase.

$|\log_2 FC| > 1$ and adjusted P value <.05. To identify upregulated DEGs, $\log_2 FC > 1$ and adjusted P value <.05 were set. To identify commonly upregulated DEGs among GSE59045 and GSE45436, E Chart online service (http://www.ehbio.com/ImageGP/index.php/Home/Index/index.html) for Venn diagram was used.

Survival Analysis

Liver Hepatocellular Carcinoma (The Cancer Genome Atlas [TCGA], Provisional) database in cBioPortal for cancer genomics web service was used for identifying potential candidate biomarkers for predicting the overall survival (OS) and disease-free survival (DFS) of patients with HCC.[17,18] Messenger RNA (mRNA) expression levels calculated by $\log_2$ calculation were compared based on clinical attribute in patients with HCC. To evaluate associations between candidate biomarkers and survival and clinicopathological features in patients with HCC, gene data with Z scores and clinical data of patients with HCC in Liver Hepatocellular Carcinoma (TCGA, Provisional) database were downloaded from cBioPortal and matched using VLOOKUP index in Excel, Microsoft Office 2016. After excluding 10 patients with liver histology of hepatocholangiocarcinoma (n = 7) and fibrolamellar carcinoma (n = 3) and 6 patients without gene expression levels, 361 patients with HCC were included in the analysis. Additionally, the Kaplan-Meier plotter online service (http://kmplot.com/analysis/)[19] was used for validation of candidates with auto select best cutoff and OS in patients with HCC.

Statistical Analysis

Differences of gene expression between the individual groups were analyzed using Mann-Whitney U test, χ^2 test, and Ridit analysis based on variables types. PASW Statistics software version 23.0 from SPSS Inc (Chicago, Illinois) was used. A 2-tailed $P < .05$ was considered significant for all tests.

Results

Identification of Commonly Upregulated DEGs in NAFLD, NASH, and HCC Tumors

Gene expression in liver of morbidly obese patients was conducted in GSE59045. We compared upregulated DEGs between patients with NAFLD/NASH and obese patients with liver histology <5% steatosis. Then we identified upregulated DEGs in tumor and nontumor tissues from patients with HCC using GSE45436 profile. As shown in Figure 1, 2 common upregulated DEGs including squalene epoxidase (SQLE) and EPPK1 were identified in NAFLD, NASH, and HCC tumors (Figure 1A). As shown in Figure 1B and C, SQLE and EPPK1 mRNA were significantly overexpressed in patients with NAFLD and NASH compared to that in obese cases <5%

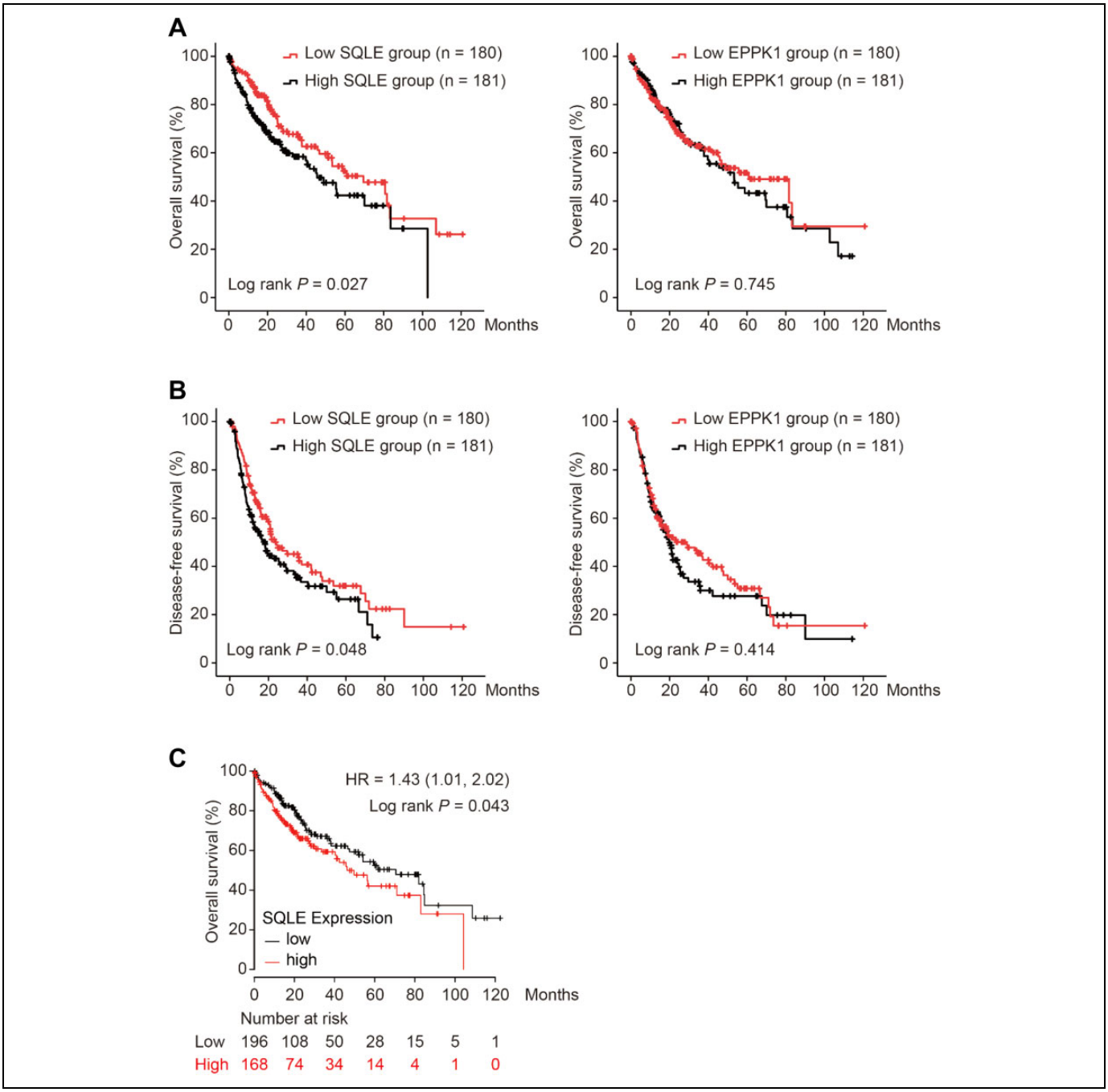

Figure 3. Comparison of overall survival (A) and disease-free survival (B) in patients with HCC grouped by SQLE and EPPK1 median cutoffs in TCGA database, and overall survival analysis for validation of SQLE was performed using Kaplan-Meier plotter (C). HCC indicates hepatocellular carcinoma; SQLE, squalene epoxidase; TCGA, The Cancer Genome Atlas.

steatosis (all $P < .01$; Figure 1B and C). As we expect, SQLE and EPPK1 mRNA were significantly upregulated in tumor tissues in patients with HCC in GSE45436, GSE60502, and GSE84402 (all $P < .01$; Figure 1D-F), which was validated in TCGA (both $P < .001$; Figure 1G). In addition, SQLE and EPPK1 mRNA were upregulated in 15.56% and 8.06% patients with HCC in TCGA profile using cBioPortal online analysis (Figure 2A). The genetic alteration and heatmap of SQLE and EPPK1 are described in Figure 2B.

Associations Between SQLE and EPPK1 and HCC Survivals

Using Liver Hepatocellular Carcinoma (TCGA, Provisional) database in cBioPortal for cancer genomics, we grouped patients with HCC with median cutoffs of SQLE and EPPK1. As shown in Figure 3, overexpression of SQLE in tumors was significantly associated with worse OS in patients with HCC (log-rank $P = .027$; Figure 3A), while no difference in OS was

Table 1. Characteristics of Patients With HCC Between SQLE High and SQLE Low Groups.

Variables	SQLE Expression Level		P Value
	Low (n = 180)	High (n = 181)	
Gender, male (%)	117 (65.0)	127 (70.2)	.294
Age, median (IQR), years	61 (18)	60 (19)	.849
BMI, kg/m^2, n (%)			.491
<18.5	13 (7.2)	8 (4.4)	
18.5-24.99	75 (41.7)	77 (42.5)	
25-29.99	42 (23.3)	46 (25.4)	
>30	38 (21.1)	29 (16.0)	
Race, n (%)			.14
Asian	70 (38.9)	86 (47.5)	
White	96 (53.3)	80 (44.2)	
Black or African American	7 (3.9)	11 (6.1)	
Tumor status, n (%)			.415
With tumor	51 (28.3)	58 (32.0)	
Tumor free	117 (65.0)	110 (60.8)	
Family history of cancer, n (%)	56 (31.1)	53 (29.3)	.705
Hepatocarcinoma risk factors, n (%)			.255
Hepatitis virus infection	50 (27.8)	61 (33.7)	
Alcohol consumption	36 (20.0)	34 (18.8)	
Hepatitis virus plus alcohol consumption	25 (13.9)	14 (7.8)	
Nonalcoholic fatty liver disease	8 (4.4)	7 (3.9)	
No risk factors	38 (21.1)	48 (26.5)	
Neoplasm histologic grade, n (%)			.036
G1	28 (15.6)	25 (13.8)	
G2	87 (48.3)	84 (46.4)	
G3	60 (33.3)	61 (33.7)	
G4	1 (0.6)	11 (6.1)	
AJCC stage, n (%)			.045
I	78 (43.3)	89 (49.2)	
II	49 (27.2)	33 (18.2)	
III	33 (18.3)	51 (28.2)	
IV	1 (0.6)	3 (1.7)	
Vascular invasion, n (%)			.362
Macro	9 (5.0)	7 (3.9)	
Micro	49 (27.2)	40 (22.1)	
None	98 (54.4)	102 (56.4)	
Child-Pugh classification, n (%)			.784
A	105 (58.3)	107 (59.1)	
B	10 (5.6)	11 (6.1)	
C	1 (0.6)	0 (0)	
NA	64 (35.6)	63 (34.8)	
AFP > 400 ng/mL, n (%)	24 (13.3)	40 (22.1)	.029
Platelet, $\times 10^3$/mm^3, n (%)			.98
<100	9 (5.0)	7 (3.9)	
100-199	62 (34.4)	51 (28.2)	
200-299	49 (27.2)	46 (25.4)	
300-399	14 (7.8)	14 (7.8)	
>400	24 (13.3)	20 (11.0)	
New tumor event after initial treatment, n (%)	46 (25.6)	47 (26.0)	.929
Ishak fibrosis status, n (%)			.676
No fibrosis	32 (17.8)	40 (22.1)	
Portal fibrosis	17 (9.4)	14 (7.8)	
Fibrous septa	13 (7.2)	15 (8.3)	
Nodular formation/incomplete cirrhosis	5 (2.8)	4 (2.2)	
Cirrhosis	39 (21.7)	31 (17.1)	
NA	74 (41.1)	77 (42.5)	
Hepatic inflammation, n (%)			.9
None	60 (33.3)	54 (29.8)	
Mild	54 (30.0)	43 (23.8)	
Severe	10 (5.6)	8 (4.4)	
NA	56 (31.1)	76 (42.0)	
Follow up, median (IQR), years	0.41 (1.8)	0.27 (1.4)	.116

Abbreviations: AJCC, American Joint Committee on Cancer; AFP, α-fetoprotein; BMI, body mass index; HCC, hepatocellular carcinoma; IQR, interquartile range; NA, not available; SQLE, squalene epoxidase.

found in EPPK1 groups (log-rank $P = .745$; Figure 3A). Moreover, high-level SQLE in tumor tissues was significantly associated with poor DFS in patients with HCC (log-rank $P = .048$; Figure 3B), and no statistical significance was observed in DFS comparison in EPPK1 groups (log-rank $P = .414$; Figure 3B). For validation, we performed OS analysis using Kaplan-Meier plotter. As shown in Figure 3C, SQLE upregulation contributed to significantly worse OS in patients with HCC (hazard ratio = 1.43, 95% confidence interval = 1.01-2.02, log-rank $P = .043$; Figure 3C).

Associations Between SQLE and Clinicopathological Characteristics in Patients With HCC

Clinicopathological characteristics comparison grouped by SQLE median are summarized in Table 1. High level of SQLE significantly contributed to advanced neoplasm histologic grade, advanced AJCC stage, and α-fetoprotein (AFP) elevation ($P = .036$, .045, and .029, respectively; Table 1). In addition, we compared SQLE mRNA expression levels based on neoplasm histologic grade, AJCC stage, and AFP level. As shown in Figure 4, SQLE mRNA was significantly overexpressed in patients with AFP >400 ng/mL and neoplasm histologic grade III-IV compared to those with AFP <400 ng/mL and neoplasm grade I-II ($P = .0002$ and $P = .0105$, respectively; Figure 4A and B). Compared to patients with HCC with AJCC stage II, those with AJCC stage III-IV had significantly higher SQLE mRNA levels ($P = .0347$; Figure 4C).

Diseases, Genes, and Functions Associated With SQLE

We searched diseases associated with SQLE in DisGeNET database. As shown in Table 2, liver neoplasm, liver carcinoma, and hypercholesterolemia were all included in the top 10 scored diseases associated with SQLE (Table 2). However, the scores were low and PMIDs were few. Therefore, more

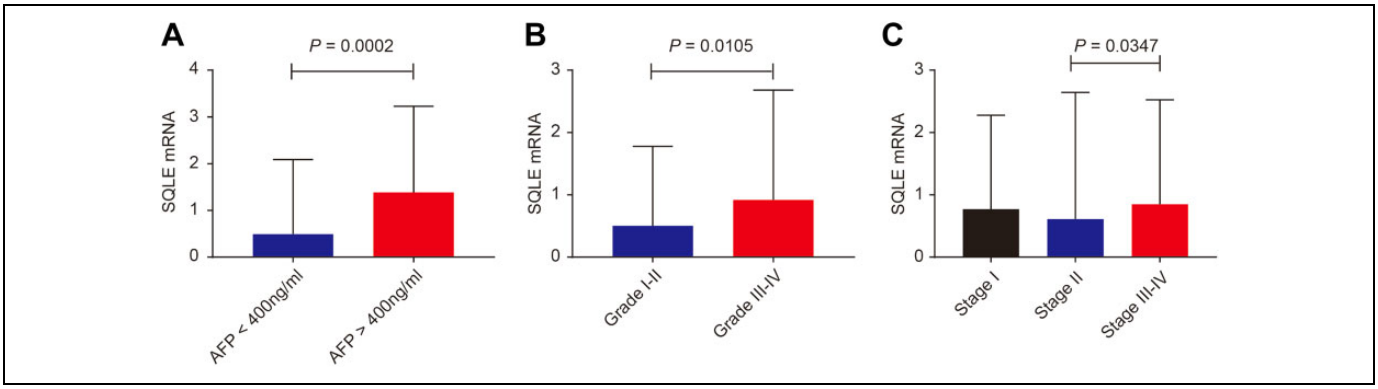

Figure 4. Squalene epoxidase mRNA levels grouped by α-fetoprotein (A), neoplasm histologic grade (B) and AJCC stage (C). mRNA indicates messenger RNA

Table 2. Top 10 Scored Disease Associations for SQLE in DisGeNET Database.

Disease	Entry Name	Score	PMIDs
C0023904	*Liver Neoplasms, Experimental*	0.2	1
C1458155	Mammary Neoplasms	0.003	1
C0006142	Malignant Neoplasm of Breast	<0.001	2
C0678222	Breast Carcinoma	<0.001	2
C0600139	Prostate Carcinoma	<0.001	2
C0376358	Malignant Neoplasm of Prostate	<0.001	2
C2239176	*Liver Carcinoma*	<0.001	2
C0020443	*Hypercholesterolemia*	<0.001	1
C1335302	Pancreatic Ductal Adenocarcinoma	<0.001	1
C1301034	Pancreatic Intraepithelial Neoplasia	<0.001	1

Abbreviation: SQLE, squalene epoxidase.

studies evaluating associations between SQLE and liver cancer and cholesterol synthesis are needed in future.

In Liver Hepatocellular Carcinoma (TCGA, Provisional) database, we searched coexpressed genes of SQLE with Spearman correlations >0.5; totally 16 genes were identified (Figure 5A). In Gene Set Enrichment Analysis database, most SQLE-coexpressed genes were enriched in Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway, GO biological process, and Reactome associated with cholesterol, sterol/steroid biosynthesis processes (Figure 5B).

Discussion

Hepatocellular carcinoma can arise in the context of noncirrhotic liver in patients with NAFLD/NASH, suggesting a specific carcinogenic pathway. Pathology studies have also described steatohepatitic HCC as a specific histological variant.[20-22] Patients are often diagnosed with HCC in the advanced NAFLD stage because of the absence of efficient surveillance policies in this population. Management of patients with HCC with NAFLD is also complicated by comorbidities, mainly cardiac disease and diabetes, which negatively affect eligibility for radical treatments.[23] Hence, finding commonly upregulated DEGs in NAFLD/NASH and HCC tumors might provide novel insights into the pathogenesis, progression, and therapeutic strategy in patients with HCC.

In our analysis, we found that SQLE and EPPK1 were commonly upregulated DEGs during chronic liver disease process including NAFLD, NASH, and HCC. Unfortunately, further analysis demonstrated that only SQLE was significantly associated with HCC survivals and clinicopathological features including AFP elevation and advanced tumor stages. Located in the endoplasmic reticulum, SQLE was found to be the one of the key rate-limiting enzymes in the cholesterol biosynthesis.[24-26] Squalene epoxidase catalyzes the first oxygenation step of the cholesterol biosynthetic pathway, the conversion of squalene to 2,3-oxidosqualene.[24,27,28] Recent studies have shown that SQLE is involved in the development and metastasis of the tumorigenesis. A report by Sui et al indicated that the expression of SQLE was upregulated in the HCC tissues. And, overexpression of SQLE in HCC cells promoted cell proliferation and migration, while downregulation of SQLE inhibited the tumorigenicity of HCC cells in vitro and in vivo. Mechanistically, SQLE positively regulated the extracellular signal-regulated kinase signaling.[29] Squalene epoxidase exerts its oncogenic effect via its metabolites, cholesteryl ester and nicotinamide adenine dinucleotide phosphate. Squalene epoxidase is expressed at very low levels in most of the noncholesterolemic tissues and is found in greatest abundance in liver. Increased SQLE expression promotes the biosynthesis of cholesteryl ester, which induces NAFLD-HCC cell growth.[30] Suppression of tumor growth by blockade of SQLE function is associated with decreased cholesteryl ester concentrations, restoration of PTEN expression, and inhibition of AKT-mTOR.[30] Considering previous study, we assumed that associated with NAFLD progression, SQLE might contribute to the disease aggressiveness of patients with HCC, especially in NAFLD/NASH population. We also suggested further basic study focusing mechanisms of SQLE in the pathogenesis and progression of NAFLD-related patients with HCC.

In breast cancer, frequent *MYC* gene substantial coamplification and aberrant methylation of SQLE promoter were observed and reduced patient survival.[31,32] Overexpression of

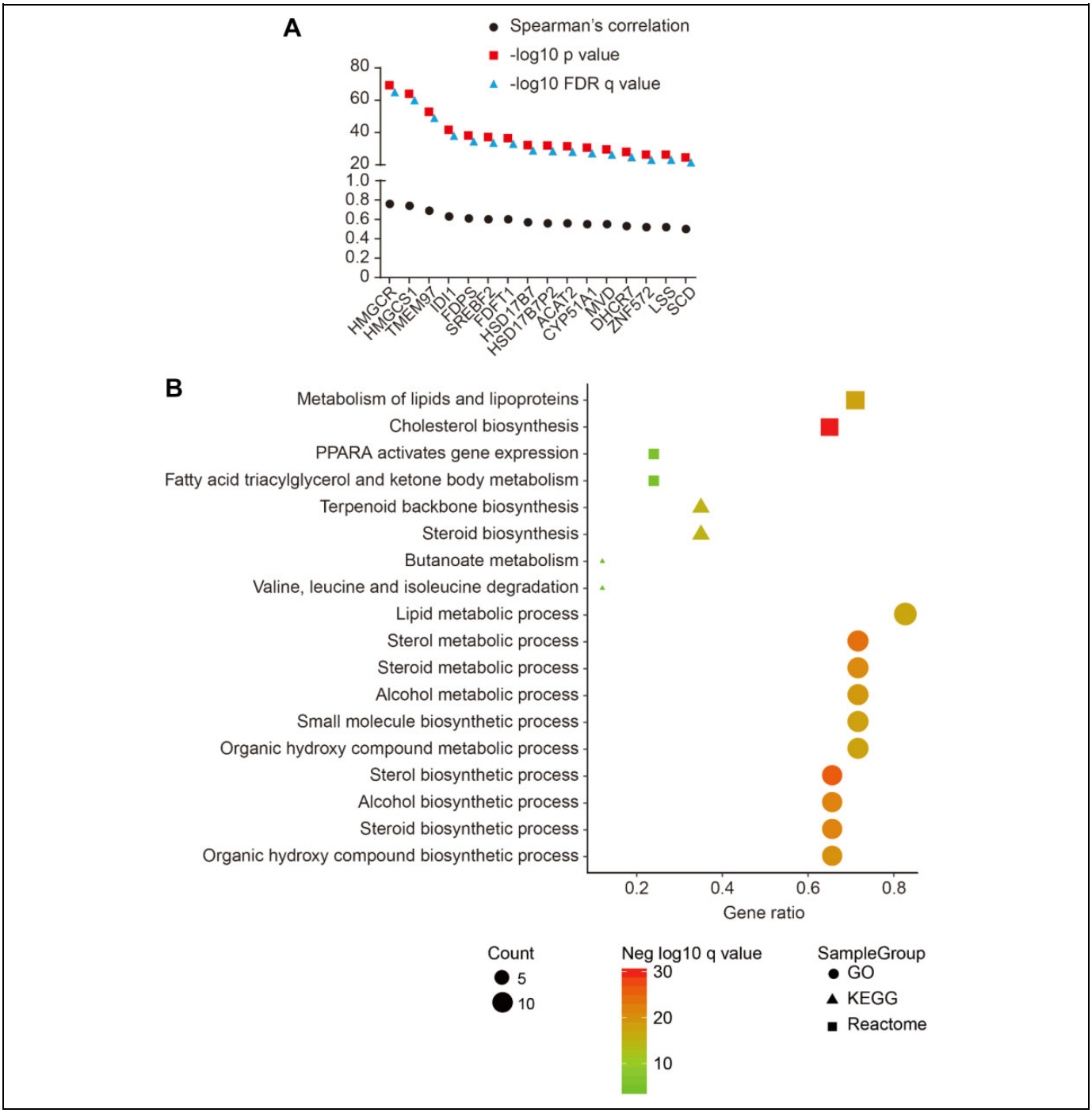

Figure 5. Coexpressed genes of SQLE with Spearman correlation >0.5 (A) and KEGG, GO, and Reactome enrichment of SQLE-coexpressed genes in GSEA (B). GSEA, Gene Set Enrichment Analysis; SQLE, squalene epoxidase.

SQLE was more prevalent in aggressive breast cancer and was an independent prognostic factor of unfavorable outcome. Inhibition of SQLE resulted in a copy-dosage correlated decrease in cell viability.[33,34] Squalene epoxidase and other genes involved in cholesterol biosynthesis were consistently associated with radioresistance in the pancreatic cancer.[35,36] In addition, low expression level of SQLE was associated with a better prognosis in patients with colorectal cancer.[37] Moreover, differential expression of SQLE was confirmed in tumor tissue in human primary lung squamous cell carcinoma and prostate acinar cancer.[38,39] The expression of SQLE mRNA was closely correlated with poor differentiation, clinical stages, lymphatic metastasis, and OS in squamous cell lung carcinoma.[40] Also, SQLE could induce epithelial-to-mesenchymal transition by regulating of miR-133b in esophageal squamous cell carcinoma.[41]

Existing literatures mainly focused on the potential mechanisms of SQLE in the promotion of NAFLD and HCC. The correlations between SQLE and HCC have not yet been illustrated. Our research was of great importance in investigating the oncogenic effects of SQLE in patients with HCC. Additionally, our findings should be considered in the context of its limitations. First, SQLE was examined in transcription levels, not in protein levels. Second, no mechanisms of these genes were conducted, such as gene silencing approaches. Based on our results and previous publications, we proposed that SQLE is an oncogene in many human malignances including NAFLD-HCC and repurposing SQLE inhibitors targeting SQLE-induced cholesterol synthesis pathway may be a promising approach for the prevention and treatment of NAFLD-HCC.[42-44]

Declaration of Conflicting Interests

The author(s) declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

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