Decreased expression levels of ELOVL6 indicate poor prognosis in hepatocellular carcinoma

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Abstract. The present study aimed to investigate the expression of elongation of very long-chain fatty acids family member 6 (ELOVL6) in hepatocellular carcinoma (HCC) tissues, and to determine its role in the development of HCC. A total of 377 HCC specimens were collected for tissue microarray and immunohistochemistry analyses. The ELOVL6 IHC score for HCC tissues was 0.97±0.71, which was significantly lower than that of the matched adjacent normal tissues (1.32±0.68; P<0.001). Patients with low levels of ELOVL6 expression were older (P=0.014) and possessed larger sized tumors (P=0.039) than patients with high expression levels. Additionally, Kaplan-Meier analysis revealed that patients with low ELOVL6 expression levels also had significantly poorer overall (P<0.001) and disease-free (P=0.029) survival times, and a greater probability of recurrence. The tumor size, tumor-node-metastasis (TNM) stage, vascular invasion and ELOVL6 expression were all shown to be prognostic variables for overall survival in patients with HCC. Multivariate analysis revealed that vascular invasion (P<0.001), TNM stage (P<0.001) and ELOVL6 expression (P=0.001) were independent prognostic variables for overall survival. In addition, vascular invasion (P=0.032) and ELOVL6 expression (P=0.041) were independent risk factors for disease-free survival, and vascular invasion (P=0.019) and ELOVL6 expression (P=0.045) were independent risk factors associated with HCC recurrence. The present study revealed that in patients with HCC, ELOVL6 expression level was reduced in HCC tissues, and that higher ELOVL6 expression levels correlated with longer survival times. This indicates that ELOVL6 may serves as an independent marker of poor patient outcome.

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Introduction

Hepatocellular carcinoma (HCC) is the most common primary malignant tumor of the liver, with a mortality rate that ranks second globally (1,2). Various etiologies of HCC have been reported, including chronic hepatitis virus infection, alcohol consumption and abnormal metabolism, and treatment is complex (3,4). Although surgical resection may be curative under certain conditions, the majority of patients are diagnosed at a late stage when surgery is no longer effective (5). A specific biomarker for HCC has yet to be identified (6,7), thus the early diagnosis of HCC and the discovery of predictive biomarkers have important clinical implications (2,8,9). Investigating the pathogenesis of HCC by identifying abnormally expressed genes or proteins may considerably improve the diagnosis and treatment of the disease.

The association between abnormal lipid metabolism and tumorigenesis has attracted increasing attention (8-11). Tumor cells have altered proliferative and metabolic abilities compared with normal cells (12,13), and the enzymes associated with fatty acid synthesis are upregulated in tumor tissues; this increases fatty acid synthesis and provides the necessary materials and energy to facilitate rapid tumor growth (14,15). Of the associated enzymes, elongation of very long-chain fatty acids family member 6 (ELOVL6) is a highly conserved member of the endoplasmic reticulase family, which is involved in the formation of long-chain fatty acids (16,17). Previous studies have found that the ELOVL6 gene serves an important role in the development and progression of breast cancer by regulating the metabolism of intracellular lipid components (18,19). Further studies have revealed that HCC cells exhibit abnormal lipid metabolism (20,21). Although the liver is the primary site of lipid metabolism (22,23), there are currently no reports of the expression and significance of ELOVL6 in HCC, a cancer closely associated with metabolic disorders. ELOVL6 is a long chain fatty acid elongase, which may contribute to fatty acid storage. Until now, the study of ELOVL6 expression in HCC has remained limited. Hence, the present study aimed to investigate the expression levels of ELOVL6 in human HCC tissues, and to determine the relationship between the expression of ELOVL6 and the prognosis of patients with HCC.

Subjects and methods

Subjects. A total of 377 paraffin-embedded HCC tissues were collected from the Jining No. 1 People's Hospital (Shandong, China) between January 2000 and July 2010. None of the patients received chemotherapy or radiotherapy prior to surgery. Patients received serological and imaging examinations to exclude recurrence or metastasis, and serum alpha-fetoprotein (AFP) level was determined. Abdomen ultrasonography, computed tomography and magnetic resonance imaging were utilized for physical examinations. Patients with missing data were excluded. The follow-up period was defined as the time interval between the date of surgery and that of death or the last follow-up. The mean follow-up interval was 30.89±28.18 months (range, 3.12-146.58 months); patient characteristics are outlined in Table I. The mean age of the patients with 48.94±12.78 (range, 28-77 years). The present study was approved by the Medical Ethics Committee of Jining No. 1 People's Hospital, and as a retrospective study, the requirement for informed patient consent was waived.

Tissue microarray (TMA) and immunohistochemistry (IHC). Following surgery, all specimens were immediately embedded in paraffin and stored at room temperature. Each tissue core (diameter, 0.6 mm) was perforated and re-embedded from the labeled area using a tissue array (MiniCore) per the manufacturer's protocol. The expression level of ELVOL6A was detected in 377 tissue-pairs (cancerous and matched-noncancerous tissues); the specimens were fixed with 4% paraformaldehyde overnight at room temperature (RT), and subsequently processed using the biotin blocking Kit (Dark, Germany) at RT for 15 min. The tissues were incubated with an anti-ELOVL6 antibody (1:1,000; cat. no. ab69857; Abcam) in a humid chamber at 4˚C overnight. The tissues were washed 3 times with PBS and incubated with biotinylated goat anti-rabbit antibodies (1:200; cat. no. S0001; Affinity Biosciences) for 1 h at 37˚C. The sections were then stained with hematoxylin at room temperature for 10 min, and observed under a light microscope (Olympus) at x4 and x20 magnification.

Semi-quantitative IHC was used to detect ELOVL6 protein expression levels according to the following intensity score criteria: 0, negative staining; 1, weak staining; 2, moderate staining; and 3, strong staining. The final scores were calculated as a percentage of positive expression multiplied by the intensity score. The median IHC score was used as a cut-off to differentiate between high and low expression levels.

Oncomine database analysis. Oncomine™ (http://www.oncomine.org) is a web-based data-mining platform aimed to facilitate novel discoveries via genome-wide expression analysis (24,25). The ELOVL6 gene was queried in the database and the results were filtered by selecting 'HCC' and 'Cancer' vs. 'Normal Analysis'. Comparisons between ELOVL6 mRNA expression levels in the HCC and adjacent normal tissues of 377 patients were analyzed using the Student's t-test.

Statistical analysis. Statistical analysis was performed using SPSS software (version 13; SPSS Inc.). The paired Student's t-test or χ^2 test was used to assess the association between ELOVL6 expression level and clinicopathological variables. A survival curve was generated using the Kaplan-Meier method (log-rank test), and the multivariate Cox proportional hazards regression model was used to assess the independence of ELOVL6 as a predictive factor for HCC. $P<0.05$ was considered to indicate a statistically significant difference.

Results

Expression of ELOVL6 in the HCC TMA. The HCC TMA (n=377) was used to determine ELOVL6 expression levels in HCC tissues. ELOVL6 was predominantly expressed in the cytoplasm of HCC cells. The ELOVL6 IHC score for HCC tissues was 0.97±0.71, significantly lower than that of matched normal tissues (1.32±0.68; $P<0.001$; Fig. 1). HCC data (Guichard Liver 2 Data) was also downloaded from the Oncomine Database (https://www.oncomine.org/resource/login.html) as validation data. These results also showed a reduction in ELOVL6 expression level in HCC tissues (supplementary Fig. 1).

Association between cytoplasmic ELOVL6 and HCC clinical features. To determine the clinical relevance of ELOVL6 expression in HCC, the association between ELOVL6 expression level and the clinical features of patients with HCC was evaluated. The median IHC score of the tumor tissues was 0.9 and a low level of ELOVL6 expression was observed in 67.9% (256/377) of the cases. The median age ($P=0.014$) and tumor size ($P=0.039$) were greater in patients with low levels of ELOVL6 expression, compared with those with high expression levels. The proportion of patients exhibiting vascular invasion was significantly higher in the low ELOVL6 expression group compared with patients in the high ELOVL6 expression group ($P=0.005$) (Table I).

Association between ELOVL6 expression level and the outcome of patients with HCC. To determine the prognostic value of ELOVL6 expression level in patients with HCC, Kaplan-Meier survival analysis was conducted using data from the 377 enrolled patients. Kaplan-Meier analysis revealed that patients with low ELOVL6 expression levels had significantly poorer overall survival times (Fig. 2A; $P<0.001$). Similarly, compared with patients with high ELOVL6 expression levels, disease-free survival time was shorter (Fig. 2B; $P=0.029$) and the probability of recurrence was higher (Fig. 2C; $P=0.044$) in those with low ELOVL6 expression levels.

Univariate and multivariate analyses of prognostic variables in HCC. To evaluate whether ELOVL6 expression was an independent risk factor for patient outcome in HCC, univariate and multivariate analyses were conducted. The tumor size, TNM stage, vascular invasion status and ELOVL6 expression were all shown to be prognostic variables for overall survival in patients with HCC. Multivariate analysis showed that only vascular invasion ($P<0.001$), TNM stage ($P<0.001$) and ELOVL6 expression ($P=0.001$) were independent prognostic variables for overall survival (Table II).

The risk factors associated with disease-free survival (Table III) and HCC recurrence were investigated further (Table IV). Univariate analysis revealed that age, TNM stage,

Table I. Clinical variables in patients with hepatocellular carcinoma exhibiting low or high ELOVL6 expression levels.

Variable	ELOVL6 expression level		P-value
	Low	High	
Sample size	256	121	
Age, years			0.014
>50	134 (52.3%)	47 (38.8%)	
≤50	122 (47.7%)	74 (61.2%)	
Sex			0.101
Male	217 (84.8%)	110 (90.9%)	
Female	39 (15.2%)	11 (9.1%)	
AFP, ng/ml			0.069
<20	45 (17.6%)	31 (25.6%)	
≥20	211 (82.4%)	90 (74.4%)	
Cirrhosis			0.670
Yes	216 (84.4%)	100 (82.6%)	
No	40 (15.6%)	21 (17.4%)	
Tumor size, cm			0.039
<5	59 (23.0%)	40 (33.1%)	
≥5	197 (77.0%)	81 (66.9%)	
Differentiation			0.347
Well-moderate	20 (7.8%)	13 (10.7%)	
Poor-undifferentiated	236 (92.2%)	108 (89.3%)	
TNM stage			0.454
I-II	112 (43.8%)	48 (39.7%)	
III-IV	144 (56.3%)	73 (60.3%)	
Vascular invasion			0.005
Yes	53 (20.7%)	15 (12.4%)	
No	203 (79.3%)	106 (87.6%)	

ELOVL6, elongation of very long-chain fatty acids family member 6; AFP, alpha-fetoprotein; TNM, tumor-node-metastasis.

vascular invasion status and ELOVL6 expression were risk factors associated with disease-free survival. Multivariate analysis showed that vascular invasion (P=0.032) and ELOVL6 expression (P=0.041) were independent risk factors associated with disease-free survival. Only vascular invasion (P=0.019) and ELOVL6 expression (P=0.045) were independent risk factors for HCC recurrence.

Subgroup analyses of the prognostic value of cytoplasmic ELOVL6 expression in patients with HCC. Stratified survival analysis was conducted to reveal the prognostic implication of ELOVL6 expression in patients with HCC. Kaplan-Meier survival analysis illustrated that ELOVL6 expression was associated with overall survival in both the TNM stage I-II (P=0.048) and stage III-IV (P=0.003) groups in patients with tumor size >5 cm (P=0.014) and those with tumor size ≤5 cm (P=0.042). ELOVL6 expression was also associated with overall survival in younger (P=0.043) and older (P=0.014) patients with HCC (Fig. 3).

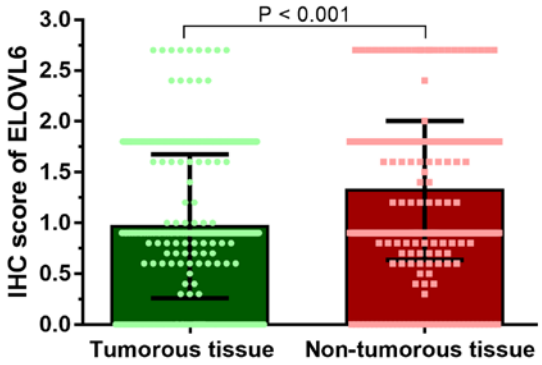

Figure 1. Expression level of ELOVL6 in HCC tissues. The ELOVL6 IHC score of 377 HCC tumor tissues was 0.97±0.71, significantly higher than that of matched normal tissues (1.32±0.68; P<0.001). ELOVL6, elongation of very long-chain fatty acids family member 6; HCC, hepatocellular carcinoma; IHC, immunohistochemistry.

Discussion

HCC is the most common malignant tumor of the liver (26-29). The result of ELOVL6 expression on the proliferation, invasion and metastasis of HCC cells was yet to be investigated, but was believed to provide insights into novel treatment options for patients with HCC. The present study confirmed that the expression level of ELOVL6 was decreased in HCC tissues, and that ELOVL6 expression was negatively associated with tumor size. Furthermore, a low expression level of ELOVL6 was associated with unfavorable outcome in patients with HCC. This indicates that ELOVL6 is a potential, novel therapeutic target and prognostic biomarker for HCC.

Fatty acids are essential components of biofilm lipids, signaling molecules and constituents of energy metabolism pathways (30-32). Among them, palmitic acid serves a prominent role in the formation of long-chain fatty acids containing 16 carbon atoms (C16:0), and studies have reported excessive accumulation of palmitic acid in breast cancer cells (33-35). ELOVL6 is a key enzyme in intracellular lipid metabolism, and has previously been associated with metabolism in fatty liver and diabetes (36). However, the relationship between metabolic reprogramming and expression of ELVOL6 in HCC has not previously been reported. In the present study, it was determined that tumor size was closely associated with ELOVL6 expression level. It is also possible that ELOVL6-associated lipid metabolism is able to promote tumor proliferation, though the specific mechanisms remain to be determined.

Moon *et al* (37) found that the conversion of palmitic acid to stearic acid (C18:0) was inhibited in ELOVL6 knock-out mice, suggesting that ELOVL6 is indispensable for palmitic acid metabolism (37). It has been speculated that ELOVL6 converts excess palmitic acid (C16:0), serving a role in tumor suppression. Kessler *et al* (38) found that in a mouse model of diethylnitrosamine-induced HCC, the expression level of ELVOL6 in cancerous tissues was lower than that in non-cancerous liver tissues. The present study was consistent with these results, where ELOVL6 expression level was also significantly reduced in HCC tissues. In addition, ELOVL6 expression was negatively associated with tumor size.

Table II. Univariate and multivariate analyses of hepatocellular carcinoma patient variables for overall survival.

	Univariate analysis			Multivariate analysis		
Variable	HR	95% CI	P-value	HR	95% CI	P-value
Age, years	0.992	0.983-1.001	0.097			
Sex	0.816	0.578-1.151	0.247			
AFP	1.097	0.835-1.442	0.505			
Cirrhosis	0.946	0.698-1.282	0.721			
Tumor size, cm	1.578	1.211-2.055	0.001			
Differentiation	1.472	0.984-2.204	0.060			
TNM stage	1.807	1.432-2.282	<0.001	1.591	1.244-2.036	<0.001
Vascular invasion	3.266	2.463-4.331	<0.001	2.678	1.992-3.600	<0.001
ELOVL6 expression	1.476	1.152-1.891	0.002	1.509	1.174-1.939	0.001

HR, hazard ratio; CI, confidence interval; AFP, α-fetoprotein; TNM, tumor-node-metastasis; ELOVL6, elongation of very long-chain fatty acids family member 6.

Table III. Univariate and multivariate analyses of hepatocellular carcinoma patient variables for disease-free survival.

	Univariate analysis			Multivariate analysis		
Variable	HR	95% CI	P-value	HR	95% CI	P-value
Age, years	0.982	0.969-0.995	0.005			
Sex	0.805	0.510-1.271	0.352			
AFP	1.244	0.852-1.817	0.258			
Cirrhosis	1.066	0.707-1.607	0.760			
Tumor size, cm	1.406	1.001-1.975	0.050			
Differentiation	1.626	0.924-2.862	0.411			
TNM stage	1.442	1.065-1.952	0.018			
Vascular invasion	1.954	1.332-2.868	0.001	1.475	1.089-1.998	0.032
ELOVL6 expression	1.441	1.036-2.004	0.030	1.478	1.062-2.058	0.041

HR, hazard ratio; CI, confidence interval; AFP, α-fetoprotein; TNM, tumor-node-metastasis; ELOVL6, elongation of very long-chain fatty acids family member 6.

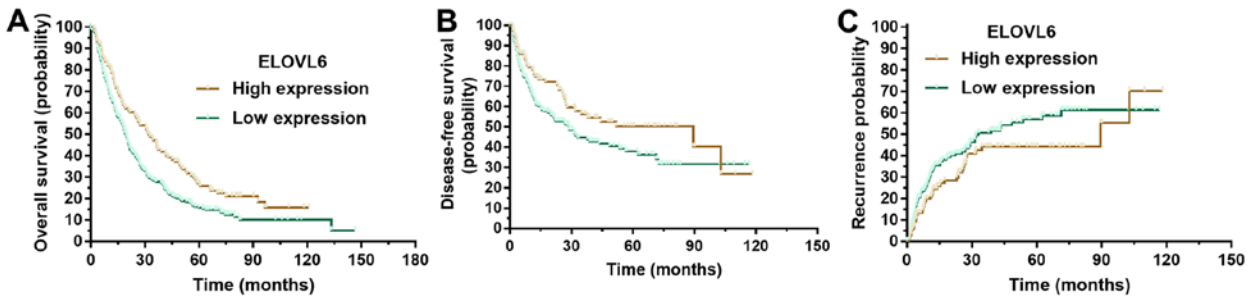

Figure 2. Prognostic values for ELOVL6 expression level in patients with HCC. Kaplan-Meier analysis revealed that (A) patients with low expression levels of ELOVL6 had significantly poorer overall survival times (P<0.001). Compared with the patients with high ELOVL6 expression levels, patients with low ELOVL6 expression had significantly (B) lower disease-free survival times (P=0.029) and (C) a higher probability of recurrence (P=0.044). ELOVL6, elongation of very long-chain fatty acids family member 6; HCC, hepatocellular carcinoma.

Previous studies have revealed that the level of palmitic and stearic acid in tumor cells was associated with the prognosis of cancer patients (39,40). Bougnoux *et al* (41) found that breast cancer patients with high levels of stearic acid in their tumors had a lower likelihood of these tumors metastasizing. Further studies also revealed that patients with breast cancer

Table IV. Univariate and multivariate analyses of hepatocellular carcinoma patient variables for recurrence.

	Univariate analysis			Multivariate analysis		
Variable	HR	95% CI	P-value	HR	95% CI	P-value
Age, years	0.836	0.612-1.143	0.262			
Sex	0.817	0.505-1.320	0.408			
AFP	1.122	0.760-1.654	0.563			
Cirrhosis	1.067	0.690-1.648	0.771			
Tumor size, cm	1.162	0.823-1.641	0.392			
Differentiation	1.432	0.811-2.528	0.215			
TNM stage	1.378	1.002-1.897	0.049			
Vascular invasion	1.839	1.217-2.778	0.004	1.773	1.196-2.674	0.019
ELOVL6 expression	1.298	1.122-1.827	0.030	1.421	1.156-1.983	0.045

HR, hazard ratio; CI, confidence interval; AFP, α-fetoprotein; TNM, tumor-node-metastasis; ELOVL6, elongation of very long-chain fatty acids family member 6.

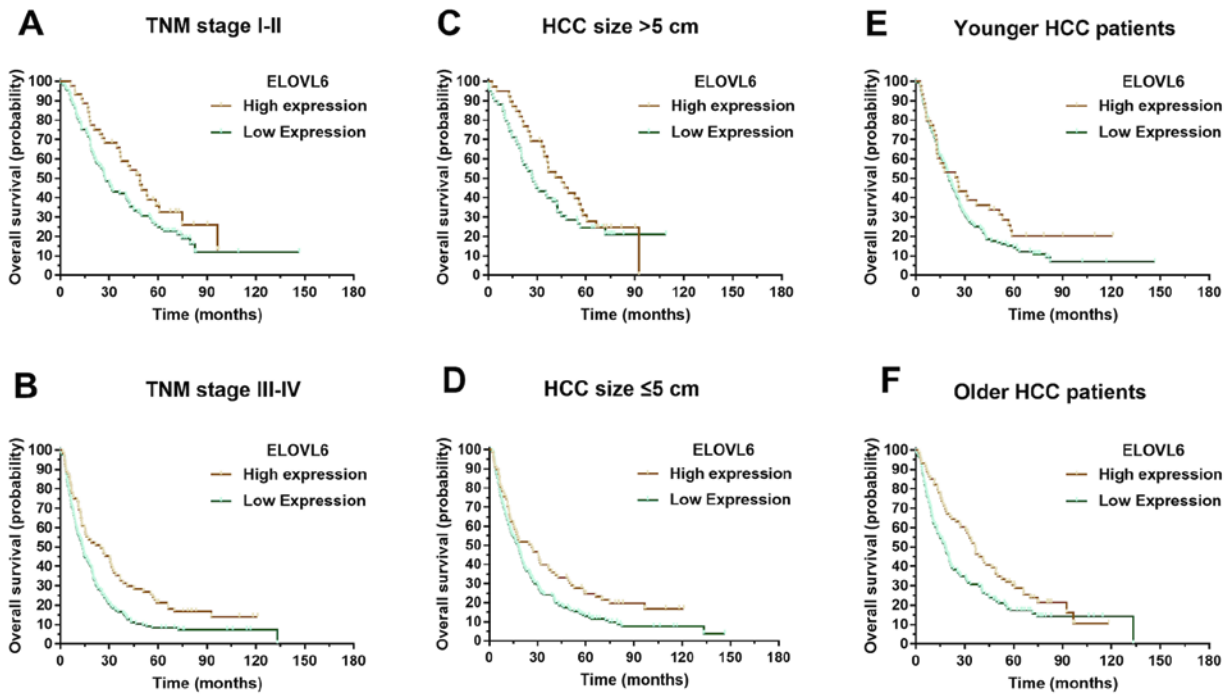

Figure 3. Prognostic prediction values of ELOVL6 expression level in subgroup analysis of patients with HCC. The overall survival between patients with high and low expression levels of ELOVL6: (A) TNM stage I-II HCCs, P=0.048; (B) stage III-IV HCCs, P=0.003. (C) In HCC size >5 cm HCCs, P=0.014; (D) ≤5 cm HCCs, P=0.042. (E) Younger patients with HCC, P=0.043; and (F) older patients with HCC, P=0.014. ELOVL6, elongation of very long-chain fatty acids family member 6; HCC, hepatocellular carcinoma.

and elevated palmitic acid levels had a poorer prognosis, and that the expression of the ELOVL6 gene was significantly downregulated in these patients (42-44). In the present study, the overall and disease-free survival time of patients with high ELOVL6 expression levels was increased. However, the hypothesis that ELOVL6 regulates intracellular lipid components and influences the prognosis of patients requires further confirmation. Lipid metabolism is a key aspect of tumor growth; fatty acids not only serve as an energy source for tumors, but as a cellular component of rapidly proliferating tumor cells. ELOVL6 extends the carbon chain of fatty acids and inhibits their use, which may be detrimental in rapid tumor proliferation. Additional studies have confirmed that ELOVL6 is involved in both migration and proliferation (45,46), and in the present study, ELOVL6 expression was associated with vascular invasion, which is also closely associated with factors such as vascular endothelial growth factor. The majority of chronic liver diseases are associated with hypoxic symptoms that come with with metabolic diseases, such as non-alcoholic fatty liver disease. Chronic hypoxia can result in the disorder of lipid metabolism and an increase in vascular endothelial growth factor expression in hepatocytes, thereby increasing

blood flow in the liver to adapt to the anoxic environment. In HCC, the formation of these microvessels also increases the migration ability of tumor cells (47,48). Vascular invasion of tumors is a complex process that utilizes the motility of tumor cells and the proliferation and migration of vascular endothelial cells (49,50). The molecular mechanism of vascular invasion is not fully explained by ELVOL6 expression; this may explain why vascular invasion was associated with ELOVL6 expression in the present study, but that they were also independent prognostic factors. It was demonstrated that the lower the expression level of ELOVL6, the higher the probability of vascular invasion, which may be due to the decreased expression level of ELOVL6 and subsequent increase in tumor cell migration. However, this theory requires further experimental confirmation.

Although ELOVL6 is involved in lipid synthesis (46), in order to meet the requirements of rapidly proliferating tumor cells, over-activated lipid-synthesized fatty acids are used to synthesize cell membranes and other organelles, rather than being stored in lipid droplets (11,51). In other tumor types, the expression of ELOVL6 was also found to be decreased (52), but in order to confirm the role of ELOVL6 in HCC, further *in vivo* and *in vitro* experimentation is required.

There are some limitations to the present study; the sample size was relatively small, which may have introduced a degree of bias. The data were also collected from a single institution, which may also have resulted in enrollment bias. A multicenter prospective study is warranted to further validate the role and potential prognostic value of ELOVL6 in HCC.

In summary, the present study highlighted a role for ELOVL6 in the development and progression of HCC. The data revealed that ELOVL6 expression level was decreased in HCC tissues, which was significantly correlated with tumor size. High ELOVL6 expression level correlated with longer survival times in patients with HCC, and therefore, ELOVL6 may serve as an independent factor for improved patient outcome. Collectively, the present study suggested that ELOVL6 may be a promising biomarker for the prognosis of patients with HCC, and a potential target for HCC treatment.

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Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Authors' contributions

MD designed the study. HL and MD wrote the manuscript. XW and JT analyzed and interpreted the patient data. HL and HZ performed the experiments. All authors read and approved the final manuscript.

Ethics approval and consent to participate

The present study was approved by the Medical Ethics Committee of Jining No. 1 People's Hospital. All procedures followed were in accordance with the ethical standards of the responsible committee on human experimentation and with the Helsinki Declaration 2008.

Patient consent for publication

As a retrospective study, the Medical Ethics Committee waived the need for informed patient consent.

Competing interests

The authors declare that they have no competing interest.

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