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Original Article

LOXL4 is downregulated in hepatocellular carcinoma with a favorable prognosis

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Abstract: Lysyl oxidase like 4 (LOXL4), a member of the secreted copper-dependent amine oxidases that contribute to the assemble and maintenance of the extracellular matrix (ECM), was found to be up-regulated or down-regulated in different cancer types, suggesting its paradoxical roles in cancer. The specific role of LOXL4 in hepatocellular carcinoma (HCC), however, is still yet to be defined. Twenty-eight pairs of HCC specimens were used for LOXL4 mRNA expression analysis. The mRNA expression in HCC cell lines was examined, and HepG2 was selected for LOXL4 small interfering RNA (siRNA) interference to investigate the biological function of LOXL4, LOXL4 immunohistochemical staining was performed using a tissue microarray containing 298 HCC patients. The prognostic and diagnostic value of LOXL4 was evaluated using Cox regression and Kaplan-Meier analysis. LOXL4 mRNA or protein expression was significantly lower in HCC tissues than peritumoral tissues (LOXL4 mRNA expression, $P = 0.018$; LOXL4 protein expression, $P < 0.001$). Low LOXL4 expression was associated with lower overall survival (OS) rates and higher cumulative recurrence rates. Multivariate analysis indicated that LOXL4 was an independent prognostic indicator for OS and time to recurrence (TTR). Our results revealed that LOXL4 was down-regulated in HCC and correlated with aggressive tumors and a worse clinical outcome. LOXL4 may be a potential biomarker to identify the HCC patients with a higher risk of recurrence.

Keywords: Lysyl oxidase like 4, hepatocellular carcinoma, immunohistochemistry, prognosis

Introduction

Hepatocellular carcinoma (HCC) is one of the most prevalent tumor types, and more than half a million patients are diagnosed worldwide annually [1]. In china, except for the heterogeneity of tumor lesions, HCC mainly derives from chronic viral hepatitis or liver cirrhosis. Despite intensive research, the prognosis of HCC remains poor, and the recurrence rate can be as high as 50% at 3 years [2]. Accordingly, the exploration of novel therapeutic approaches is urgent.

The lysyl oxidase (LOX) family consists of secreted copper-dependent amine oxidases and is comprised of five paralogues: LOX and LOX-like 1-4 (LOXL1-4). The essential function of the LOX family members is to catalyze the cross-linking of collagens and elastin in the ECM, maintaining ECM homeostasis [3]. A member of the LOX family, LOXL4 shares two domains: a conserved C-terminal copper binding and catalytic domains, and a unique N-terminal domains, which determines individual roles [4]. A study from Hayahi et al. revealed that the LOX family is localized both intra- and extracellularly in different areas of various tissues from normal, young adult mice [5], suggesting that the LOX family may have different functions. Weise et al. reported that LOXL4 expression was upregulated in head and neck squamous cell carcinomas (HNSCC), and was associated with high-grade dysplasia and lymph node metastases [6]. The overexpression of LOXL4 correlated with hypoxia in colorectal cancer [7] and gastric cancer [8], however, by inhibiting the Ras/ERK signaling pathway in bladder cancer, LOXL4 acted as a tumor suppressor gene [9]. To date, LOXL4

expression and the association with clinicopathological factors in HCC remains unclear.

In our study, the LOXL4 mRNA expression level was detected from a set of 28 HCC tumor tissues and peritumoral tissues using real-time PCR. Then, we examined the LOXL4 protein expression level using a tissue microarray containing 298 HCC patients by IHC staining procedures, and the clinical significance of LOXL4 expression was studied. Finally, we evaluated the prognostic significance of LOXL4 in HCC patients.

Materials and methods

Patients and specimens

Tissue specimens were obtained from consecutive patients with HCC who underwent curative resection at the Liver Cancer Institute, Zhongshan Hospital, Fudan University, between 2007 [10]. Briefly, the histopathological diagnosis was determined according to the World Health Organization criteria. Tumor differentiation was graded using the Edmondson grading system [11]. Tumor staging was based on the 6th edition of the tumor-node-metastasis (TNM) classification of the International Union Against Cancer. Ethical approval was obtained from the Zhongshan Hospital Research Ethics Committee, and written informed consent was obtained from each patient.

Follow-up and postoperative treatment

In brief, the follow-up data were summarized at the end of December 2012, with a median observation time of 52.2 months. The follow-up procedures were described in our previous study [11, 12]. Postsurgical patient surveillance was undertaken as previously described [11, 13]. The overall survival (OS) was defined as the interval between the dates of surgery and death. The time to recurrence (TTR) was defined as the interval between the dates of surgery and the dates of any diagnosed recurrence (intrahepatic recurrence and extrahepatic metastasis). For surviving patients, the data were censored at the date of death or last follow-up.

HCC cell lines, cell transfection, RNA extraction, cDNA synthesis and qRT-PCR

The HCC cell lines, Cell Transfection, RNA extraction, cDNA synthesis and qRT-PCR was described previously [14, 15]. The following primers were used:

GAPDH: 5'-AAGGTGAAGGTCGGAGTCAAC-3' and 5'-GGGGTCATTGATGGCAACAATA-3',

LOXL4: 5'-CTGGGCACCACTAAGCTCC-3' and 5'-CTCCTGGATAGCAAAGTTGTCAT-3'.

The sequences of LOXL4 siRNA are as follows: LOXL4 siRNA1: 5'-GGUGCAAUGUCCCUAACAUTT-3', NC sequence: 5'-UUCUCCGAACGUGUCACGUdTdT-3'.

Immunohistochemistry and quantitative analysis

Immunohistochemistry staining was carried out as previously described [14]. Briefly, after microwave antigen retrieval, the slides were preincubated with primary antibodies against LOXL4 overnight, followed by an incubation with secondary antibodies, and treatment with horseradish peroxidase-conjugated streptavidin. The sections were incubated in a 3, 3-diaminobenzidine solution, counter stained with hematoxylin, dehydrated in ethanol, cleared in xylene, and cover slipped. The negative controls were treated in all of the assays (with the omission of primary antibodies). Quantification of the LOXL4 expression level was evaluated by a computer assisted image system [16, 17]. Briefly, two images, representing duplicate sample, were captured using Leica QWin Plus v3 software at a magnification of 100 (Leica Microsystems Imaging Solutions Ltd). The integrated absorbance and the area were analyzed using Image-Pro Plus v6.0 software (Media Cybernetics, Inc.). Finally, the mean LOXL4 density was calculated as the ratio of integrated absorbance/total area. The X-tile software (Yale University) [18] was used to define the optimal cut-off values for LOXL4 protein levels, which is applied elsewhere [19-21].

Statistical analysis

All analyses were performed with SPSS software (version 19.0, SPSS, Chicago, IL). Comparisons of quantitative data were analyzed using Student's t test between two groups or by one-way ANOVA for multiple groups. The X^2 test or Fisher's exact test was used for categorical data. The Kaplan-Meier method was used to determine survival probability, and dif-

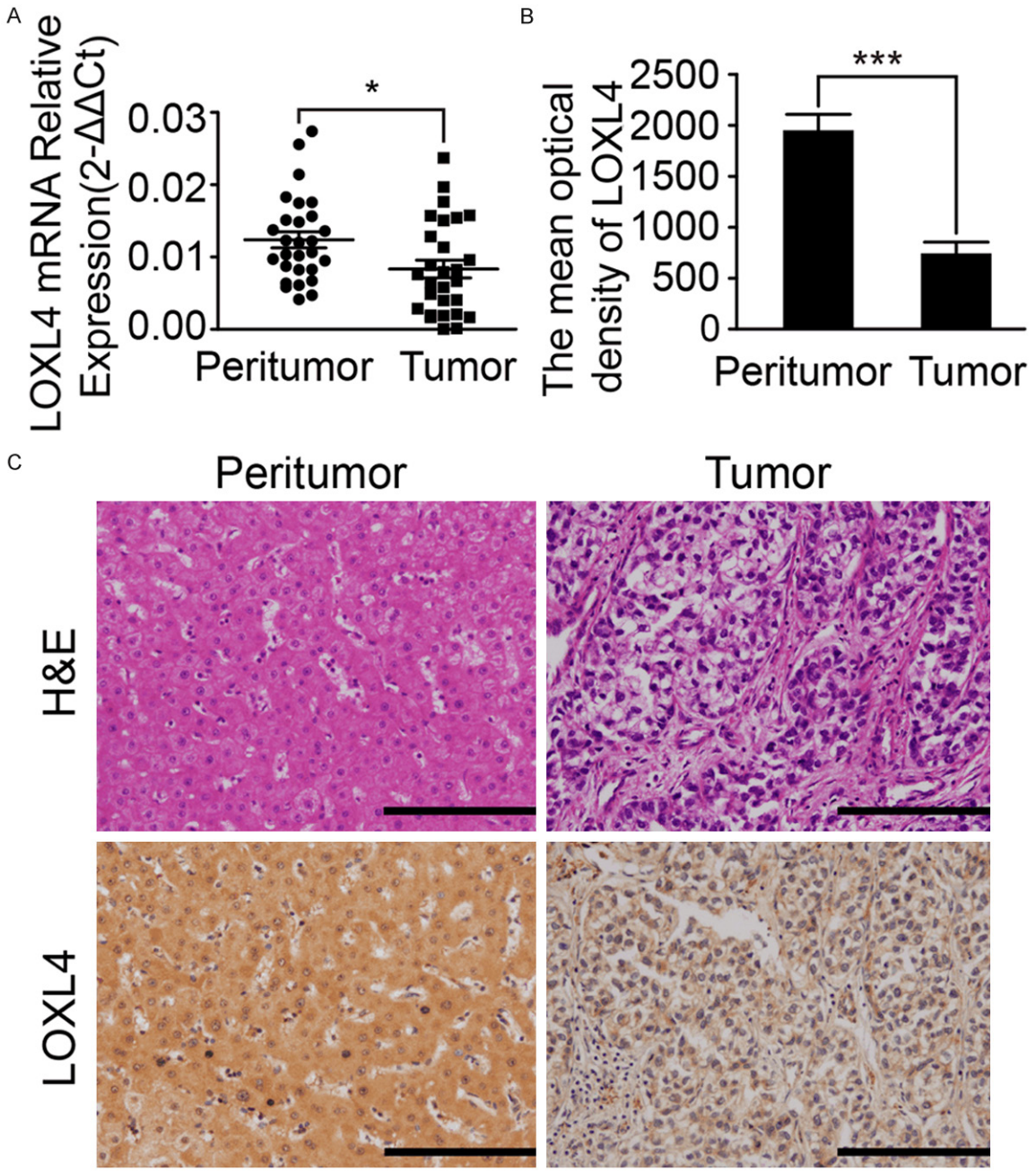

Figure 1. Analysis of LOXL4 expression in HCC tissues. A. Analysis of LOXL4 mRNA expression in paired samples of tumor and adjacent non-cancerous liver tissue. Statistical analysis by paired *t* test (n = 28; **P* < 0.05). B. Post IPP analysis showed the significantly difference of LOXL4 protein expression between tumor and adjacent tumor tissues. Statistical analysis by paired t test (n = 298; ****P* < 0.0001). C. Representative LOXL4 expression in HCC tumors and non-tumor tissues by immunohistochemistry on the tissue microarray and corresponding HE staining results. Scale bar 50 μm.

ferences were calculated with the log-rank test. A Cox proportional hazard regression model was used for univariate and multivariate analyses, and multivariate analysis was performed using a forward, stepwise Cox regression model. Two-tailed *p* values < 0.05 were considered statistically significant.

Results

Analysis of LOXL4 mRNA expression by qRT-PCR

To examine the LOXL4 expression level in clinical samples, we applied qRT-PCR analysis on

Table 1. Correlation of clinicopathological characteristics with LOXL4 expression

Variable	LOXL4 protein level (n=298)		
	$LOXL4^{Low}$	$LOXL4^{High}$	*P* value†
Age (years)			
≤ 51 yr	74	61	0.910
> 51 yr	98	83	
Sex			
Male	24	14	0.299
Female	148	130	
γ-GT (units/L)			
≤ 54	73	70	0.307
> 54	99	74	
AFP (ng/ml)			
≤ 20	66	61	0.491
> 20	106	83	
HBsAg			
Negative	37	25	0.395
Positive	135	119	
Liver cirrhosis			
No	32	18	0.164
Yes	140	126	
Microvascular invasion			
absence	120	98	0.807
present	52	46	
Tumor size			
≤ 5 cm	106	102	0.096
> 5 cm	66	42	
Tumor differentiation			
I+II	125	104	1.000
III+IV	47	40	
Multinodular tumor			
Single	149	118	0.277
Multiple	23	26	
Tumor encapsulation			
Complete	86	77	0.573
None	86	67	
TNM stage			
I-II	110	80	0.135
III-IV	62	64	
BCLC stage			
A	94	91	0.137
B, C	78	53	

Abbreviations: HBsAg, hepatitis B surface antigen; AFP, α-fetoprotein; γ-GT, γ-glutamyl transferase; TNM, tumor-nodes-metastasis; *Serum samples obtained from patients were used for ELISA validation. † A *P*-value < 0.05 was considered statistically significant. *P*-values were calculated using the Pearson chi-square test.

paired tumor samples with corresponding normal tissues. Twenty-eight paired human tumor and peritumoral samples from HCC patients were collected. The expression of LOXL4 was normalized to the expression of GAPDH in each sample. As is shown in **Figure 1A**, the levels of LOXL4 mRNA were significantly decreased when compared with paratumor tissue specimens (0.008 ± 0.001 vs. 0.012 ± 0.001, $P = 0.018$). In line with the Oncomine data, the average LOXL4 mRNA expression was significantly lower in HCC compared with liver cancer precursor lesions ($P < 0.001$, data not shown).

Expression of LOXL4 protein in HCC tissue

Haematoxylin and eosin staining revealed that the neoplastic cells were relatively homogeneous within the tumor tissue. Then, we examined LOXL4 expression in primary tumors composed of 298 HCC patients by immunohistochemical (IHC) staining. Most of the stromal cells were negatively stained, although sporadic positive staining of these cells was observed. IHC staining revealed that the staining pattern of LOXL4 was mainly cytoplasmic in both the tumor and peritumoral tissues (**Figure 1C**). In contrast with the $LOXL4^{Low}$ group, the $LOXL4^{high}$ group accounted for 42.95% (119/298) of the total patients. IPP software analysis confirmed that LOXL4 expression was significantly downregulated in tumors in comparison with their counterparts (the mean density of LOXL4 protein, 741.8 ± 110.8 vs. 1954 ± 153.0, $P < 0.001$, **Figure 1B**). There was no significant relationship between LOXL4 and histopathological tumor grade. The correlations of LOXL4 expression with the clinicopathologic characteristics are shown in **Table 1**. Clinical characteristics were not directly related to the expression of LOXL4.

LOXL4 did not correlate with HCC cell lines growth rate

Next, we examined LOXL4 mRNA expression in a series of HCC cell lines with stepwise metastatic potential (Hep3B, HepG2, SMMC7721, Huh7, MHCC97L, MHCC97H, and HCCLM3). The mRNA expression trend of LOXL4 in cancer cells was in contrast with their metastatic potential ($P < 0.05$, **Figure 2A**). Then, HepG2 was chosen and subjected to RNA interference

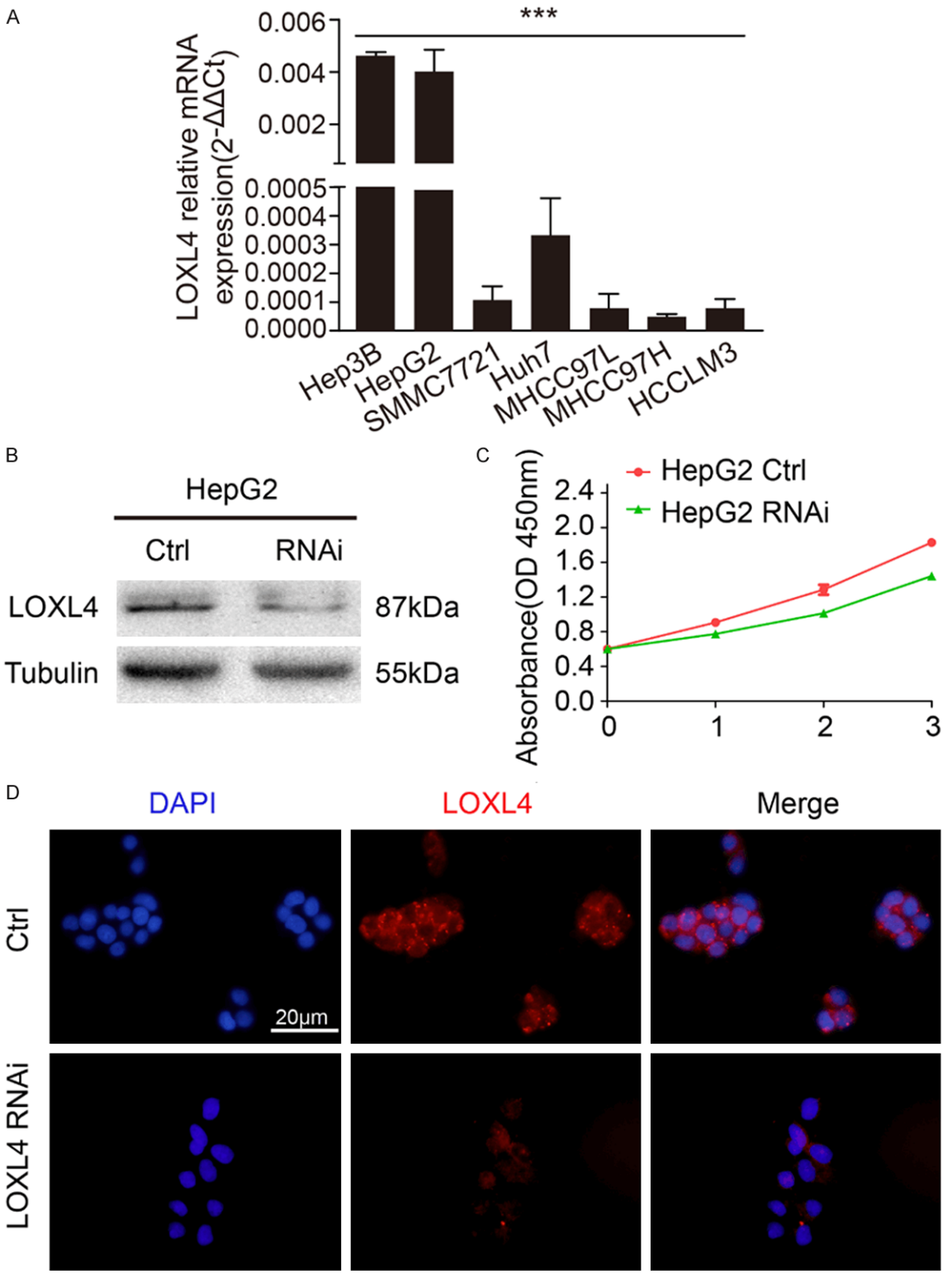

Figure 2. Knockdown of LOXL4 in HepG2 doesn't correlate with the growth rate. A. LOXL4 mRNA expression trend in HCC cell line. qRT-PCR result showed that LOXL4 mRNA expression level decreased with the increase of metastatic potential in HCC cell line. B. LOXL4 expression in HepG2 was modified by LOXL4 siRNA and verified by western blot. C. Cell proliferation was detected by CCK-8 assay. D. Immunofluorescence staining demonstrated that LOXL4 expression level was down-regulated in HepG2 LOXL4 siRNA cell lines. Scale bar 20 μm.

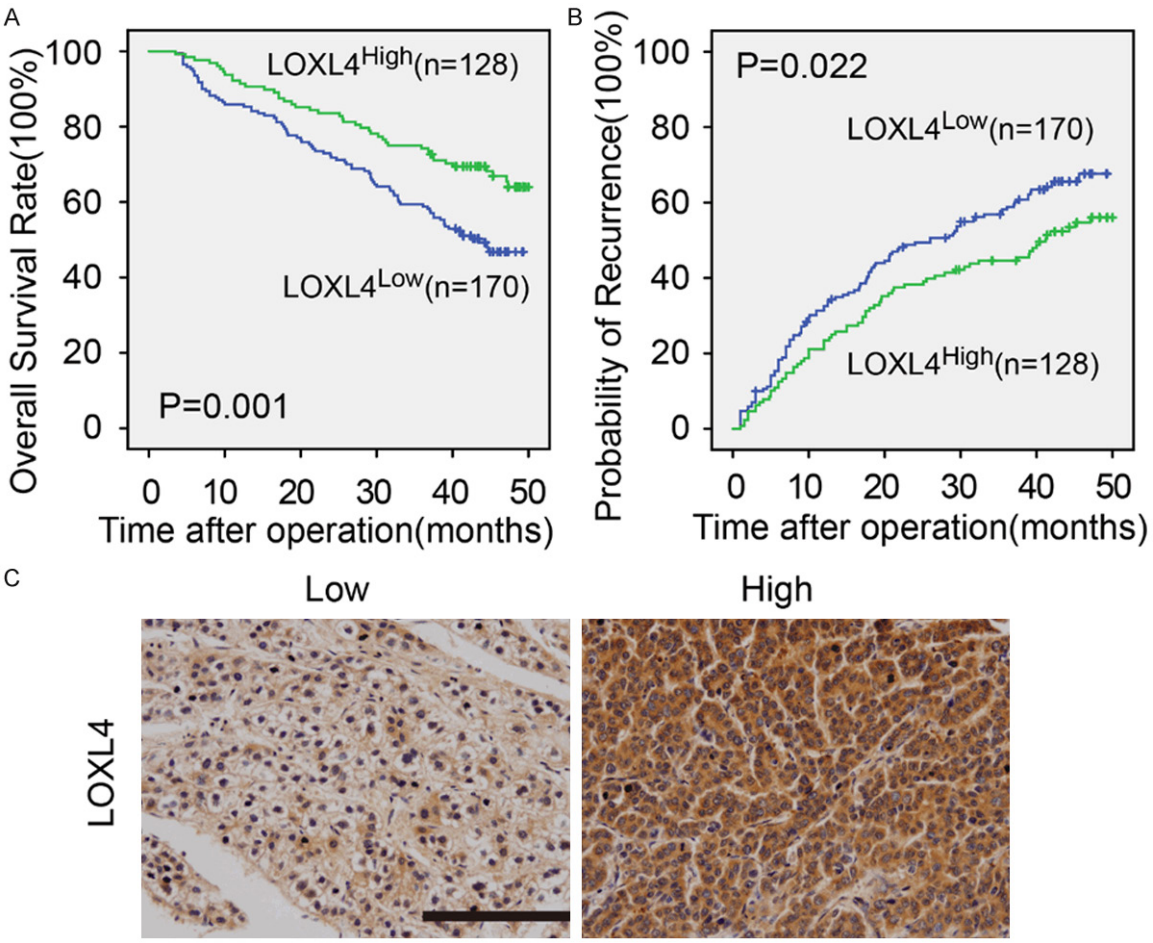

Figure 3. Kaplan-Meier analysis of OS and TTR for the LOXL4 expression level in 298 cases of HCC patients. IHC showed the low and high expression levels of LOXL4 in HCC. Scalebar applies to all images, 200×, 50 μm.

for LOXL4. LOXL4 siRNA treatment induced a clear down-regulation at the protein level, which was also confirmed by immunofluorescence staining (**Figure 2B**, **2D**). In contrast with the control group, LOXL4 siRNA did not attenuate the growth of HepG2 cell lines, suggesting that LOXL4 did not have an effect on the growth of HCC cell lines (**Figure 2C**).

LOXL4 down-regulation indicated poor prognostic in HCC patients

By the last follow-up (December 2012), 59.7% (178/298) of the patients had suffered from recurrence and 43.6% (130/298) had died. The 1-, 3-, and 5-year OS rates in the whole cohort were 88.3%, 65.8%, and 56.4%, respectively, and the 1-, 3- and 5-year cumulative recurrence rates were 28.5%, 51.7%, and 59.7%, respectively. Additionally, we found that the 1-, 3-, and 5-year survival rates of the $LOXL4^{High}$ patients were significantly higher than those of the $LOXL4^{Low}$ group (91.4% vs. 85.9%, 74.2% vs. 59.4%, and 66.4% vs. 48.8%, respectively). Similarly, the $LOXL4^{Low}$ patients had a poorer prognosis at 1-, 3-, and 5- years, with higher cumulative recurrence rates than the $LOXL4^{High}$ patients (32.4% vs. 23.4%, 57.1% vs. 44.5%, and 64.1% vs. 53.9%, respectively) (**Figure 3A**, **3B**).

Univariate analysis of patient survival and recurrence was carried out based on clinicopathological parameters. LOXL4 was associated with OS and TTR (P = 0.001, P = 0.022, respectively). γ-GT, tumor size, microvascular invasion, tumor differentiation, AFP and TNM stage were predictors of OS and TTR. HBsAg was only associated with TTR. The individual clinicopathological features that presented significance in the univariate analysis were adopted as covariates in a multivariate Cox propor-

Table 2. Univariate and multivariate analyses of prognostic factors associated with survival

Variables	OS			
	Univariate	Multivariate		
	P value	*P* value	HR	95% CI
TMA assays (n=298)				
Sex (female vs. male)	0.704	NA		
Age, years (≤ 50 vs. > 50)	0.917	NA		
HBsAg (negative vs. positive)	0.093	NS		
AFP, ng/ml (≤ 20 vs. > 20)	0.009	NS		
γ-GT, U/L (≤ 54 vs. > 54)	0.006	NS		
Liver cirrhosis (no vs. yes)	0.991	NA		
Tumor size, cm (≤ 5 vs. > 5)	0.000	0.000	3.542	2.437~5.149
Tumor number (single vs. multiple)	0.189	0.005	1.916	1.221~3.007
Encapsulation (complete vs. none)	0.078	NA		
Microvascular invasion (no vs. yes)	0.001	NS		
Tumor differentiation (I-II vs. III-IV)	0.012	0.014	1.598	1.100~2.321
TNM stage (I vs. II III)	0.001	NS		
LOXL4 (Low vs. High)	0.001	0.003	0.568	0.392~0.823

Abbreviations: OS, overall survival; AFP: α-fetoprotein; γ-GT, γ-glutamyl transferase; TNM, tumor-nodes-metastasis; HR, hazard ratio; CI, confidential interval; NA, not adopted. Boldface type indicates significant values. †Cox proportional hazards regression. a. Degree of freedom reduced because of constant or linearly dependent covariates.

tional hazards model for further analysis. LOXL4 was a meaningful prognostic indicator of OS and TTR ($P = 0.003$, $P = 0.043$, respectively) (**Table 2**).

Discussion

To date, this is the first study investigating the relationship between LOXL4 expression level and clinicopathological features of HCC tumor and peritumoral tissues. We showed that LOXL4 was significantly down-regulated in HCC tissues. Moreover, we revealed that the down-regulation of LOXL4 expression was correlated with higher recurrence rates and lower survival rates after curative resection.

Recently, research indicated that LOXL4 up-regulation antagonized Ras by activating the extracellular signal-regulated kinase (ERK) signaling pathway in bladder cancer, which maybe a candidate suppressor gene [9]. Simultaneously, other studies directly demonstrated the cancerous suppressor role for LOXL4 depending on in *vitro* and in *vivo* model systems. A study by Asuncion et al. [22] revealed LOXL4 is expressed in liver and prostate samples, but not in prostate and breast carcinomas. These findings are consistent with our results in this study, in which LOXL4 mRNA and protein expression was significantly lower in HCC tumor tissues in contrast to their counterparts.

In our experiments, we initially investigated LOXL4 mRNA expression in HCC tissue and their counterparts and showed down-regulation in neoplastic tissues. IHC staining was also performed to detect LOXL4 protein expression levels. Our results showed that low LOXL4 correlated with higher recurrence rate and lower overall survival rate.

Others have reported that LOXL4 is selectively upregulated in head and neck cancer [6, 23, 24], especially in nodal metastases compared to primary tumors. According to the large series of clinical samples in our study, LOXL4 was closely correlated with OS and TTR. Importantly, the observation that different gene family members play various active roles in different tissues is not uncommon, due to the specific environment and different upstream signals.

In recent years, additional evidence suggests that epigenetic alterations are an essential molecular mechanism contributing to the inactivation of tumor suppressor genes in cancer [25]. A study from Wu et al. found that LOXL1 and LOXL4, were frequently silenced in human bladder cancer, which was predominantly related to promoter hypermethylation. These findings led to the hypothesis that both genetic and epigenetic mechanisms in cancer progression were likely involved in the regulation of LOXL4 gene expression, however, the methylation status of the LOXL4 gene in HCC remains to be elucidated.

In summary, evidence from this research demonstrated that, compared with peritumoral specimens, LOXL4 expression in HCC was extremely down-regulated. Low LOXL4 expression was closely associated with lower OS rates and higher cumulative TTR rates.

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Disclosure of conflict of interest

None.

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