ORIGINAL RESEARCH

Overexpression of LBH is associated with poor prognosis in human hepatocellular carcinoma

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Purpose: Limb-bud and heart (LBH) levels are correlated with adverse survival in several malignancies; however, their significance in hepatocellular carcinoma (HCC) remains unclear. The objective of this study was to determine the association between LBH status and clinical outcomes.

Methods: We selected 226 patients with HCC who were treated surgically between 2003 and 2010 at a single academic center. Immunohistochemistry (IHC) was used to detect the protein expression of LBH in HCC samples. Receiver operating characteristic (ROC) curve analysis, Spearman's rank correlation, Kaplan–Meier plots, and the Cox proportional hazards regression model were used to analyze the data.

Results: A high expression of LBH was detected in 20 (8.8%) of 226 HCC samples. Correlation analysis demonstrated that LBH in HCC was significantly correlated with aspartate amino-transferase (AST)/alanine aminotransferase (ALT) levels and clinical stages ($P<0.05$). In the Kaplan–Meier analysis, the mean survival time of patients with low levels of LBH was longer than that for those with high levels of LBH ($P<0.05$). The 3-year overall survival rate was 20% for patients with HCC and high levels of LBH versus 67% for patients with HCC and low levels of LBH. In the multivariate analysis, AST/ALT level, clinical stage, tumor relapse, and the level of LBH were the independent prognostic factors for overall survival ($P<0.05$).

Conclusion: Overexpression of LBH might contribute to the development and progression of HCC. LBH could be a novel prognostic marker for HCC.

Keywords: LBH, hepatocellular carcinoma, immunohistochemistry, prognosis

Introduction

The 2017 cancer statistics report showed that over the past 25 years, the total cancer mortality in the US has fallen by 25%; however, the incidence and mortality of hepato-cellular carcinoma (HCC) are increasing. HCC is a deadly cancer that occurs three times more frequently in men than in women, partly reflecting higher levels of hepatitis B and C virus infection rates, historical rates of smoking, and alcohol consumption in men.[1,2] Surgery is the main radical treatment for HCC; however, 60%–70% of patients with HCC relapse within 5 years after radical resection.[3] HCC treatment outcomes are affected by several variables, including liver function and clinical stage.[4] Studies on the etiology of HCC defined viral infections, obesity, and diabetes as factors for HCC.[5,6] However, the genes driving the development of HCC need to be fully elucidated.

The limb-bud and heart (LBH) protein acts as a key transcriptional regulator in the mitogen-activated protein kinase signaling pathway to mediate cellular functions.[7,8] LBH was isolated from a human embryonic heart cDNA library. The LBH cDNA is 2,927 bp long, encoding a protein product of 105 amino acids. Al-Ali et al[9] reported that, similar to other intrinsically disordered proteins (IDPs), LBH may undergo

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a "disorder-to-order" transition upon binding to its target molecules and depending on the target-induced conformational changes, might acquire different functional activities. Rieger et al first noted an association of LBH with cancer in 2010, observing that the *LBH* gene played an important transcriptional role in wingless/int-1 (Wnt) signaling pathways, which suppress mammary epithelial cell differentiation and could contribute to Wnt-induced tumorigenesis. Thus, LBH was highlighted as a potential new marker for therapeutically challenging basal-like breast cancer.[7] However, there has been no relevant report on the clinicopathological significance and mechanism of LBH in HCC. In the present study, we used immunohistochemistry (IHC) to evaluate the protein level of LBH in HCC and statistical analysis methods to identify the association between LBH and the prognosis of HCC.

Methods
Patients and tissue specimens

In this study, 226 patients with HCC treated surgically between 2003 and 2010 who were patients of the Department of Pathology of Sun Yat-sen University Cancer Center (Guangzhou, China) were selected. Case selection was based

on the pathological diagnosis of HCC, including complete follow-up data. These HCC cases included 203 (89.8%) men and 23 (10.2%) women. Average follow-up time was 60.23 months (median: 58.5 months; range: 1.0–185 months). In all, 47 patients (20.8%) were diagnosed at late stages (III and IV) and the other 179 patients (79.2%) were diagnosed at early stages (I and II). Patients whose cause of death remained unknown were excluded from our study. Table 1 details the patients' clinicopathological features (age, gender, history of hepatitis, alpha-fetoprotein [AFP], cirrhosis, tumor size, clinical stage, and relapse). Tumor differentiation was based on the Edmondson and Steiner criteria. Tumor stage was defined according to the tumor–node–metastasis (TNM) classification system proposed by the American Joint Committee on Cancer/International Union Against Cancer.

IHC

Immunohistochemical staining of the LBH protein was detected using the standard EnVision procedure. The paraffin-embedded tissue blocks were cut into 3-μm thick sequential sections. The slides were dried and deparaffinized in xylene,

Table 1 Correlation of LBH expression with patients' clinicopathological features in HCC

Variable	LBH protein			
	All cases, n	Low expression, n (%)	High expression, n (%)	*P*-value[a]
Age (years)				0.473
<51[b]	107	96 (89.7)	11 (10.3)	
≥51	119	110 (92.5)	9 (7.5)	
Gender				0.115
Male	203	183 (90.1)	20 (9.9)	
Female	23	23 (100)	0 (0)	
HbsAg				0.156
Positive	207	187 (90.3)	20 (9.7)	
Negative	19	19 (100)	0 (0)	
AFP (ng/mL)				0.061
<81[c]	112	106 (94.6)	6 (5.4)	
≥81	112	98 (87.5)	14 (12.5)	
Liver cirrhosis				0.301
No	90	80 (88.9)	10 (11.1)	
Yes	127	118 (92.9)	9 (7.1)	
Tumor size (cm)				0.200
<5.5[d]	109	102 (93.6)	7 (6.4)	
≥5.5	115	102 (88.7)	13 (11.3)	
AST/ALT				0.042*
<1.4[e]	115	109 (94.8)	6 (5.2)	
≥1.4	111	97 (87.4)	14 (12.6)	
Relapse				0.272
No	138	131 (94.9)	7 (5.1)	
Yes	66	60 (90.9)	6 (9.1)	
Clinical stage				0.027*
I–II	179	167 (93.3)	12 (6.7)	
III–IV	47	39 (83.0)	8 (17.0)	

Notes: [a]Chi-square test. [b]Mean age. [c–e]Mean. *$P<0.05$.
Abbreviations: LBH, limb-bud and heart; HbsAg, hepatitis B surface antigen; HCC, hepatocellular carcinoma; AFP, alpha-fetoprotein; AST, aminotransferase; ALT, alanine aminotransferase.

rehydrated through graded alcohol, immersed in 3% hydrogen peroxide for 10 min to block endogenous peroxidase activity, and subjected to antigen retrieval by pressure cooking for 3 min in citrate buffer (pH =6.0). The slides were then incubated with 5% bovine serum albumin (BSA) for 15 min to reduce nonspecific reactions. Subsequently, the slides were incubated with a rabbit anti-LBH polyclonal antibody (ab122223, 1:400 dilution; Abcam, Cambridge, MA, USA) for 50 min at 37°C. The slides were sequentially incubated with a secondary antibody (K5007; Dako Denmark A/S, Glostrup, Denmark) for 30 min in the incubator at 37°C and stained with 3,3′-diaminobenzidine (DAB). Finally, the sections were counterstained with Mayer's hematoxylin, dehydrated, and mounted. A negative control was obtained by replacing the primary antibody with normal rabbit IgG.

IHC evaluation

LBH protein levels were evaluated by microscopic examination of the stained tissue microarray (TMA) slides. Each TMA spot was ranked using an intensity evaluation from 1 to 3 (*A*) and with the proportion of tumor cells as 5% increments from a range of 0–100 (*B*). A final score (range 0–300) was achieved using each intensity and the proportion of the area stained (score = $A \times B$).[10] LBH expression was scored by three independent senior pathologists who were blinded to the patients' clinicopathological data. Their diagnoses were completely consistent in ~75% of the cases, which proved that this method was highly repeatable. If two or all of them agreed with the diagnosis results, the value was selected. If their opinions were different, all three of them repeated the diagnosis and came to a consensus by discussion.

Statistical analysis

SPSS software (version 21.0; SPSS Inc., Chicago, IL, USA) was used for the statistical analysis. Receiver operating characteristic (ROC) analysis was performed to determine the cutoff value for LBH. The correlation between LBH protein levels and clinicopathological parameters in patients with HCC was analyzed using the Chi-square test. The survival of patients with HCC was evaluated using the Kaplan–Meier method with the log-rank test. Multivariate analyses were performed using the Cox proportional hazards model. All *P*-values were reported by two-sided analyses, and *P*<0.05 was considered as statistically significant.

Ethics approval and consent to participate

This study was approved by the Institute Research Medical Ethics Committee of Sun Yat-sen University Cancer Center.

No informed consent (written or verbal) was obtained for use of retrospective data from the patients within this study, most of whom were deceased, since this was not deemed necessary by the ethics committee, who waived the need for consent. All samples were anonymized.

Results
Expression of LBH in HCC

The IHC results showed that LBH protein was mainly located in the nuclei in HCC (Figure 1) and that 8.8% (20/226) of the HCC specimens were positive for LBH.

Selection of a cutoff value for LBH expression

ROC curves were used to determine the cutoff score for positive expression of the LBH protein. The sensitivity and specificity for liver cirrhosis, tumor stage, and survival status were plotted. The ROC curve analysis revealed that the cutoff value for the expression of LBH protein was 85 (area under the curve [AUC] =0.591, *P*=0.018; Figure 2).

Association of LBH expression with HCC patients' clinicopathological parameters

Chi-square analysis revealed that a high level of LBH was more common in patients with higher aspartate aminotransferase (AST)/alanine aminotransferase (ALT) levels (*P*=0.042) and those at later clinical stages (*P*=0.027). There was no significant correlation between LBH levels and other clinicopathological parameters, such as patient age, gender, hepatitis history, liver cirrhosis, AFP level, tumor size, and tumor relapse (*P*>0.05). The details are shown in Table 1.

Relationship between clinicopathological features, LBH levels, and patient survival: univariate survival analysis

Univariate survival analysis demonstrated a significant effect of well-known clinicopathological prognostic parameters, such as tumor size (*P*=0.001), serum AST/ALT level (*P*=0.002), clinical stage (*P*=0.004), and relapse (*P*=0.000). LBH expression (*P*=0.000) could be used as an important factor to assess the prognosis of HCC. Kaplan–Meier survival analysis showed that the mean survival time of the low-LBH group was significantly longer than that of the high LBH group (111.4 months vs 30.2 months, *P*=0.000; Table 2 and Figure 3). Among the 206 patients with HCC and low LBH levels, the 3-year mean survival rate was 67%; however, in the high LBH group (20 patients), the mean 3-year survival rate was only 20% (Table 3).
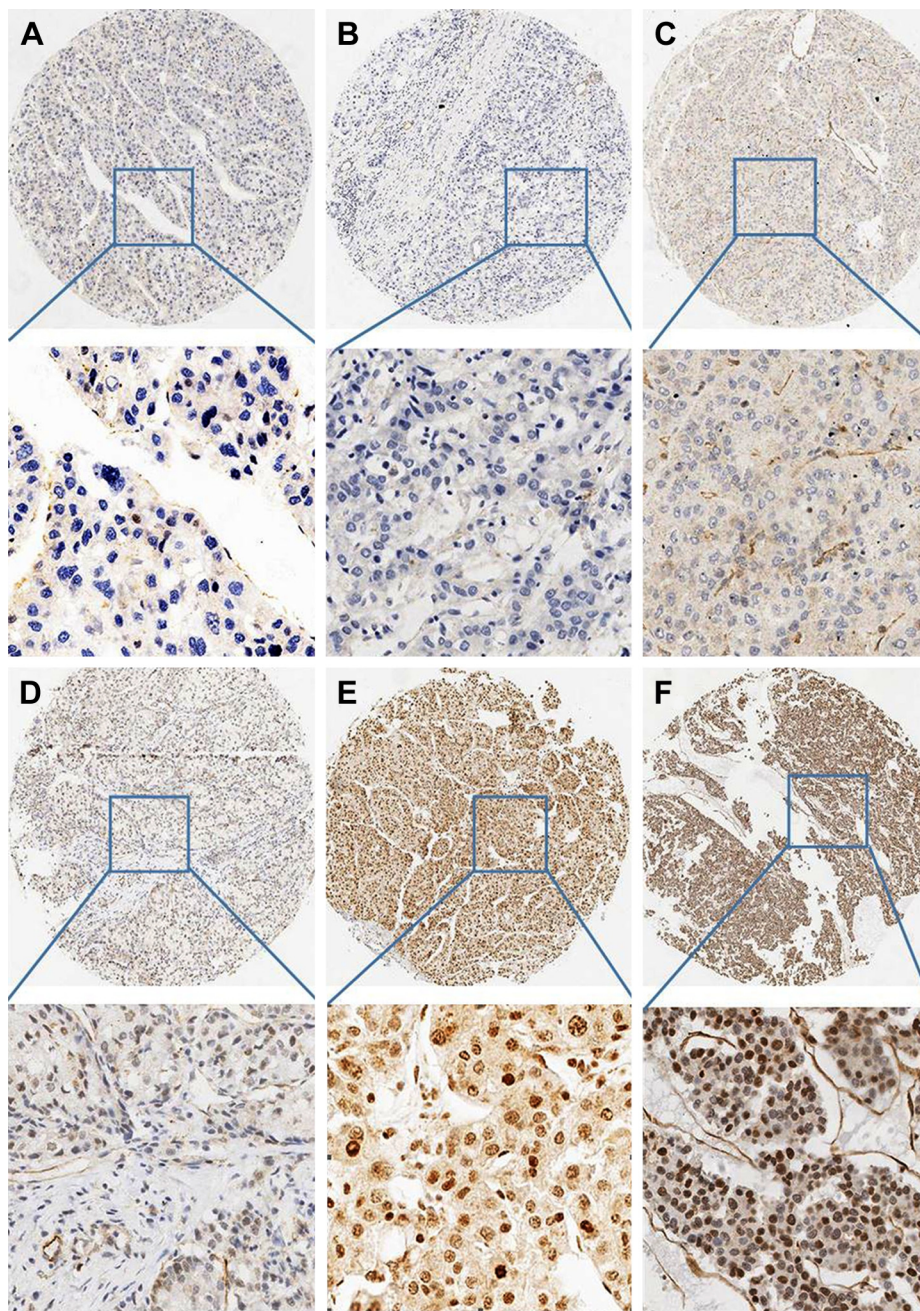
Figure 1 Expression of LBH in HCC.
Notes: (**A** and **B**) HCC cases showing nearly negative expression of LBH protein (magnification ×4, ×20). (**C** and **D**) HCC cases demonstrating low expression of LBH (magnification ×4, ×20). (**E** and **F**) High expression of LBH in HCC (magnification ×4, ×20).
Abbreviations: LBH, limb-bud and heart; HCC, hepatocellular carcinoma.

Further analysis was performed with regard to LBH levels in subsets of patients at different stages of HCC. The results demonstrated that a high level of LBH was a prognostic factor in patients with stage I ($P=0.022$), stage II ($P=0.001$), and stage III ($P=0.000$) HCC. However, they could not differentiate the outcome of patients with stage IV HCC ($P=0.095$). The details are shown in Figure 4.

Independent prognostic factors of HCC: multivariate Cox regression analysis

The factors that had a significant difference in the univariate analysis were subjected to Cox regression analysis. The results showed that a high level of LBH was an independent prognostic factor associated with poor overall patient survival (hazard ratio [HR]: 3.075; 95% confidence interval
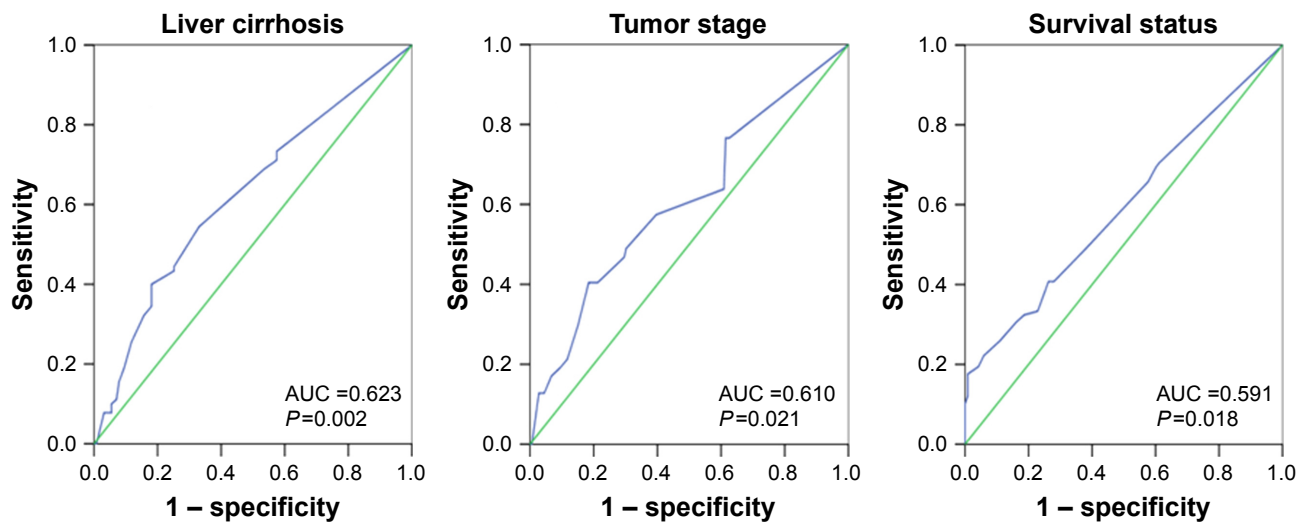
Figure 2 ROC curves were used to determine the cutoff score for positive expression of the LBH protein.
Note: The sensitivity and specificity for each outcome were plotted: liver cirrhosis, tumor stage, and survival status.
Abbreviations: ROC, receiver operating characteristic; LBH, limb-bud and heart; AUC, area under the curve.

Table 2 Univariate analysis of LBH expression and various clinicopathological parameters in 226 patients with HCC (log-rank test)

Variable	All cases, n	Mean survival (months)	P-value
Age (years)			0.52
<51	107	80	
≥51	119	105	
Gender			0.348
Male	203	99.6	
Female	23	86.7	
HbsAg			0.744
Positive	207	100.3	
Negative	19	82.8	
AFP (ng/mL)			0.161
<81	112	86.4	
≥81	112	92.7	
Liver cirrhosis			0.193
No	90	96.6	
Yes	127	85.8	
Tumor size (cm)			0.001*
<5.5	109	93.7	
≥5.5	115	87	
AST/ALT			0.002*
<1.4	115	92.4	
≥1.4	111	84.2	
Relapse			0.000*
No	138	123.6	
Yes	66	67.2	
Clinical stage			0.004*
I–II	179	108.4	
III–IV	47	62.7	
LBH expression			0.000*
Low	206	111.4	
High	20	30.2	

Note: *$P<0.05$.
Abbreviations: LBH, limb-bud and heart; HbsAg, hepatitis B surface antigen; HCC, hepatocellular carcinoma; AFP, alpha-fetoprotein; AST, aminotransferase; ALT, alanine aminotransferase.

[CI]: 1.644–5.749, $P=0.000$; Table 4). At the same time, the study also found that the serum AST/ALT level ($P=0.004$), tumor relapse ($P=0.001$), and clinical stage ($P=0.012$) were independent prognostic factors for overall patient survival.

Discussion

LBH is a member of a highly conserved family of small acidic proteins in vertebrates, which are localized at the nucleus.[11]
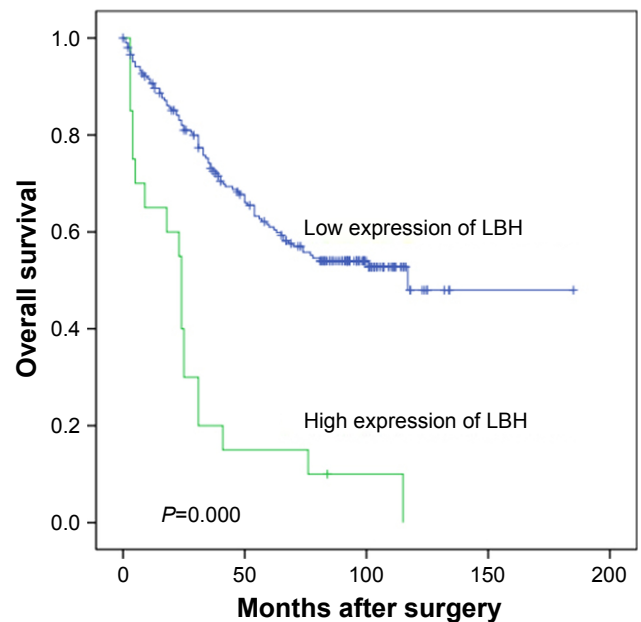

Figure 3 Kaplan–Meier survival analysis of LBH expression in total patients.
Notes: Probability of survival of all patients with HCC: low expression, n=206; high expression, n=20.
Abbreviations: LBH, limb-bud and heart; HCC, hepatocellular carcinoma.

Table 3 Three-year survival rate in 226 patients with HCC

Variable	All cases, n	Survival <3 years, n (%)	Survival >3 years, n (%)	*P*-value
LBH expression				0.000*
Low expression	206	68 (33)	138 (67)	
High expression	20	16 (80)	4 (20)	

Note: *P<0.05.
Abbreviations: HCC, hepatocellular carcinoma; LBH, limb-bud and heart.

LBH is a key transcriptional regulator, with pivotal roles in embryonic development, early mouse heart development, and human diseases.[9,12] However, the molecular mechanisms upstream of LBH and its role in tumor development remain unknown.

In the present study, we found that high levels of LBH could be detected in 20/226 (8.8%) of HCC samples. Correlation analysis demonstrated that LBH protein levels in HCC were significantly correlated with serum AST/ALT levels and clinical stage. In the Kaplan–Meier analysis, the mean survival time of patients with low levels of LBH was longer than that of those with high levels of LBH. The results suggested that the LBH level might be a new treatment classification for HCC patients.

In the multivariate analysis, serum AST/ALT level, tumor stage, tumor relapse, and the LBH level were the independent prognostic factors for overall survival. Indeed, Rieger et al[7] also found that LBH was aberrantly overexpressed in mammary
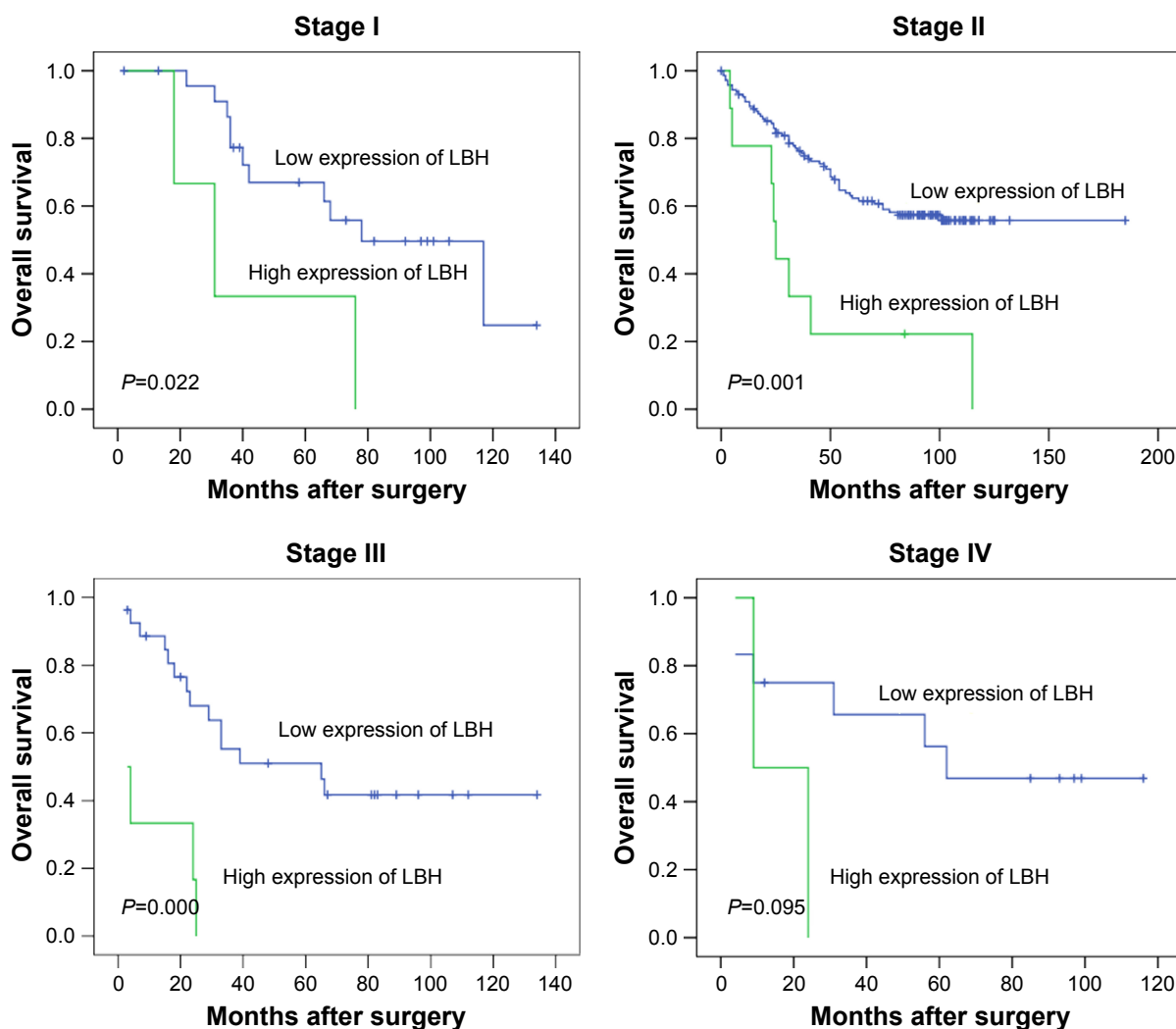

Figure 4 Kaplan–Meier survival analysis of LBH expression in subsets of patients at different clinical stages (log-rank test).
Notes: Stage I: low expression, n=24; high expression, n=3. Stage II: low expression, n=143; high expression, n=9. Stage III: low expression, n=27; high expression, n=6. Stage IV: low expression, n=12; high expression, n=2.
Abbreviation: LBH, limb-bud and heart.

Table 4 Cox multivariate analyses of prognostic factors for overall survival

Variable	HR	95% CI	P-value
AST/ALT (≥1.4 vs <1.4)	1.884	1.222–2.905	0.004*
Tumor size (≥5.5 cm vs <5.5 cm)	1.374	0.893–2.113	0.149
Clinical stage (III–IV vs I–II)	1.848	1.143–2.990	0.012*
Relapse (yes vs no)	2.102	1.346–3.285	0.001*
LBH expression (high vs low)	3.075	1.644–5.749	0.000*

Note: *$P<0.05$.
Abbreviations: HR, hazard ratio; CI, confidence interval; AST, aminotransferase; ALT, alanine aminotransferase; LBH, limb-bud and heart.

tumors of MMTV-Wnt1 transgenic mice, as well as in highly aggressive basal-like subtypes of human breast cancers. This result is consistent with ours. However, the study of Liu et al[13] showed that LBH was considerably downregulated in nasopharyngeal cancer tissues. LBH functions as a tumor suppressor of nasopharyngeal carcinoma by inducing G1/S cell cycle arrest. Another study showed that overexpression of LBH significantly inhibited cell growth in fibroblast-like synoviocytes.[14] The conclusion from these studies is that LBH can function as both an oncogene and a tumor suppressor gene.

Ai et al reported that overexpression of LBH activated the transcriptional activities of activator protein 1 (AP-1; also known as JUN) and serum response element (SRE), which are potential targets of extracellular signal-regulated kinase (ERK), JUN N-terminal kinase (JNK), and p38 in cellular signaling and functions. LBH might act as a transcriptional activator in mitogen-activated protein kinase signaling pathways to mediate cellular functions.[8] LBH is the specific receptor of transforming growth factor beta (TGF-β) and is an essential factor to promote TGF-β function.[13,15,16] LBH could reduce the expression of cyclin-dependent kinase (CDK) to affect cell proliferation.[13] A primary breast cancer study showed that LBH expression was highest in the Claudin low subtype and a higher LBH expression correlated with worse survival.[16,17] Deregulation of LBH in human basal breast cancer appears to be Wnt/b-catenin dependent, as Dickkopf WNT signaling pathway inhibitor 1 (DKK1) and Wnt7a inhibit LBH expression in breast tumor cells. Overexpression studies indicated that LBH suppresses mammary epithelial cell differentiation, an effect that could contribute to Wnt-induced tumorigenesis.[7] Collectively, LBH induces the expression of key epithelial stem cell transcription factors. These studies identified LBH as an essential regulator of basal mammary stem cell expansion/maintenance, raising important implications for its potential role in breast cancer pathogenesis.[18] This is consistent with our findings. Tumor cells that overexpressed LBH were chaotically multiplying, and the prognosis of this group of patients was poor.

The 3-year overall survival rate was 20% for the high LBH group versus 67% for the low LBH group. Taken together, our clinical data and expression studies provided evidence that LBH could be used as an independent prognostic factor to assess the overall survival of patients with HCC.

Conclusion

Overexpression of LBH is associated with poor prognosis in human HCC. LBH could be used as an independent prognostic factor in patients with HCC and is expected to be a molecular target for anticancer therapy.

Acknowledgment

Author contributions

YX and JWC were responsible for the study design. JWC, CQH and KC performed the experiments and drafted the manuscript; SML, XZ, JC and MYC participated in the data analysis and interpretation. All authors read and approved the final manuscript. All authors contributed toward data analysis, drafting and critically revising the paper and agree to be accountable for all aspects of the work.

Disclosure

The authors report no conflicts of interest in this work.

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