Original Article

DOI: 10.5582/bst.2016.01123

# The significance of low levels of LINC RP1130-1 expression in human hepatocellular carcinoma

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**Summary** **Hepatocellular carcinoma (HCC) is the most common neoplasms. Little progress has been made in the diagnosis and treatment of HCC and its prognosis remains poor. Studies have increasingly found that long non-coding RNA (lncRNA) is involved in the regulation of the occurrence and development of HCC. To investigate the diagnostic and prognostic value of lncRNA in HCC, the current study examined 25 lncRNAs with differing levels of expression (according to the fold change) in microarray databases. Expression of LINC RP1130-1 was found to be markedly down-regulated in 51 HCC tissues compared to matching adjacent non-tumor liver tissues. The pattern of expression and clinical significance of LINC RP1130-1 were examined in HCC. The area under the receiver operating characteristic (ROC) curve was 0.74 for LINC RP1130-1. The expression of LINC RP1130-1 was associated with clinical stage, the number of tumors, portal vein tumor thrombus (PVTT), and microvascular invasion (MVI). More importantly, patients with a low level of LINC RP1130-1 expression had a shorter recurrence-free survival (RFS) ($n = 51$, $p < 0.05$) than those with a high level of LINC RP1130-1 expression. Taken together, these findings indicate that a low level of LINC RP1130-1 expression in patients with HCC may be a powerful tumor biomarker, with potential clinical use in diagnosing and predicting the prognosis for patients with HCC.**

***Keywords:*** LncRNA, hepatocellular carcinoma, biomarker

## 1. Introduction

Hepatocellular carcinoma (HCC) is the most common neoplasms, accounting for approximately 90% of liver cancer, and liver cancer is currently the second leading cause of cancer-related death worldwide (*1,2*). The main risk factors for developing HCC are well-known and include cirrhosis, hepatitis B virus (HBV) or hepatitis C virus (HCV) infection, alcohol abuse, aflatoxin B1 ingestion, and non-alcoholic steatohepatitis. Major pathways with frequent mutations in HCC include the telomere maintenance pathway, the cell cycle pathway, the WNT-β-catenin pathway, the epigenetic remodeling pathway, and the chromatin remodeling pathway (*3-9*). Despite progress in the diagnosis and treatment of HCC, its prognosis still remains unfavorable. The median survival rate for curative therapy remains approximately 50% for 5 years. Recent studies have sought to ascertain the mechanisms underlying the initiation, propagation, and development of HCC in order to identify potential diagnostic biomarkers and therapeutic targets (*10-13*). Important work is to identify novel biomarkers for early diagnosis and evaluation of the prognosis of patients with HCC.

Released online in J-STAGE as advance publication October 24, 2016.

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Long non-coding RNA (lncRNA) is RNA that is longer than 200 nucleotides in length and that does not code for proteins, although lncRNA can interact with proteins (*14,15*). lncRNA is not as well characterized as other small non-coding RNA such as microRNA, but lncRNAs play important roles in the regulation of a variety of cellular processes, including stem cell pluripotency, cell growth, cell proliferation, apoptosis, metabolism, and cancer cell migration(*16-21*). Functional lncRNA may be useful in cancer diagnosis and evaluation of prognosis and it may serve as a potential therapeutic target. Studies have reported that aberrant lncRNA expression affects tumor cell growth, apoptosis, invasion, and metastasis. The lncRNA MALAT1, a highly conserved LncRNA expressed in the nucleus, should allow prediction of lung cancer development, metastasis of prostate cancer, and presenting signs of esophageal squamous cell carcinoma (*22-24*). The lncRNA UCA1 plays a key role in human bladder cancer growth and tumorigenesis; UCA1may have crucial biological activity and it may serve as a new therapeutic target for bladder cancer (*25*). The lncRNA BANCR has been found to offer potential as a diagnostic marker of lung cancer (*26*), Zfas1 offers potential as a marker of breast cancer (*27*), and TUG1 offers potential as a diagnostic marker of bladder cancer (*28*). In addition, the lncRNAs PVT1 (*29*), HULC (*30*), HEIH (*31*), ATB (*32*), DANCR (*33*), and LINC00152 (*34*) have been found to be dys-regulated in conditions like HCC.

The present study examined microarray data from human lncRNA in public databases. The datasets GSE55191 (*www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE55191*) and GSE58043 (*www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE58043*) were obtained to analyze differences in levels of lncRNA expression in HCC and paired non-tumor tissues. Twenty-five lncRNAs with differing levels of expression (according to the fold change) in the datasets were examined (Figure 1A). The level of expression of these lncRNAs was determined in a normal human liver cell line LO2 and the HCC cell lines HepG2 and SMMC-7721 (Figure 1B). Four 4 lncRNAs that varied the most in their fold change in the HepG2 and SMMC-7721 cell lines and the LO2 cell line were consistent with the results from the microarray. As shown in Figure 1B, lncRNA L12, L17, L18, and L24 exhibited an impressive fold change (>10-fold). Expression of these lncRNAs was examined in 18 paired adjacent non-cancerous hepatic tissues (Figure 2). Results indicated that LINC RP1130-1 had the greatest difference in expression among the 4 lncRNAs. LINC RP1130-1 is located on gene RP11-30J20.1 (ENSG00000254101), which is the source of numerous lncRNA transcripts. The correlation between levels of LINC RP1130-1 expression and clinicopathological characteristics and recurrence-free survival (RFS) was examined in patients with HCC to determine whether

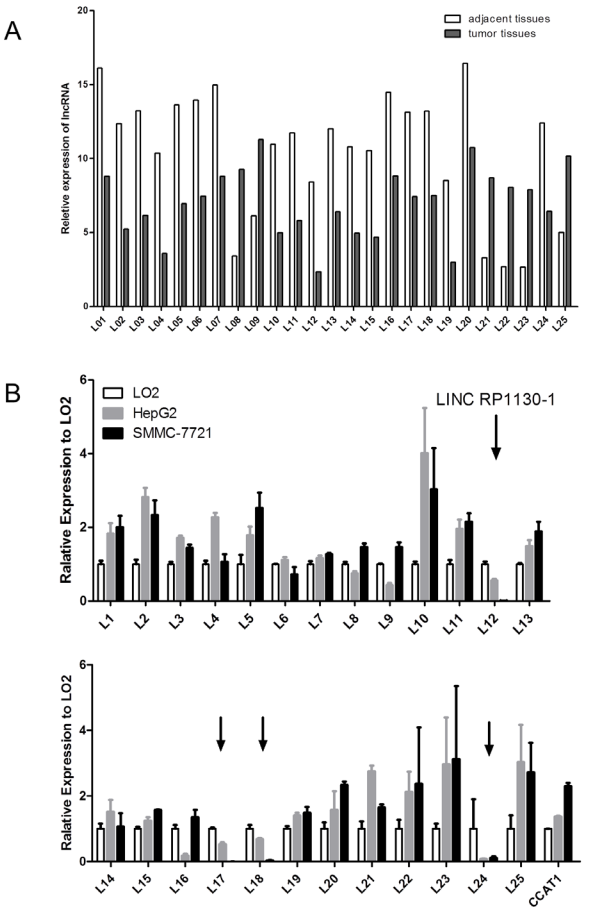


**Figure 1. Twenty-five lncRNAs with differing levels of expression were identified from datasets (GSE55191 and GSE58043) and (A) the level of their expression in a normal human liver cell line (LO2) and HCC cell lines (HepG2 and SMMC-7721) and human HCC tissues was determined (B).** L01-L25 respectively represent ASHG19A3A-012552, 018493, 054627, 006377, 051778, 024516, 054629, 054311, 054103, 053906, 015954, 040795, 014006, 021193, 025198, 033235, 043138, 044877, 047009, 053783, 054945, 027421, 044247, 029103, and 010811 in the datasets. "↓" represents 4 lncRNAs that were chosen to measure their level of expression. L12 represents LINC RP1130-1 (a positive control) and CCAT1 represents lncRNA CCAT1 (another positive control) that is reported to be consistently up-regulated in HCC cell lines. Gene numbers L17, L18, and L24 in the Ensembl database were respectively ENSG00000255723.1, ENSG00000251138.2, and ENSG00000269353.1.

LINC RP1130-1 could be a useful diagnostic and prognostic indicator in HCC.

## 2. Materials and Methods

### 2.1. *Clinical specimens and cell lines*

Data on 51 consecutive patients (42 males and 9 females) who underwent surgery for HCC at Hospital 302 in Beijing between August 2013 and April 2016 were collected from the records of the hospital's Department of Hepatobiliary Surgery. None of the patients had received preoperative chemotherapy or radiation therapy. All HCC diagnoses were confirmed histopathologically

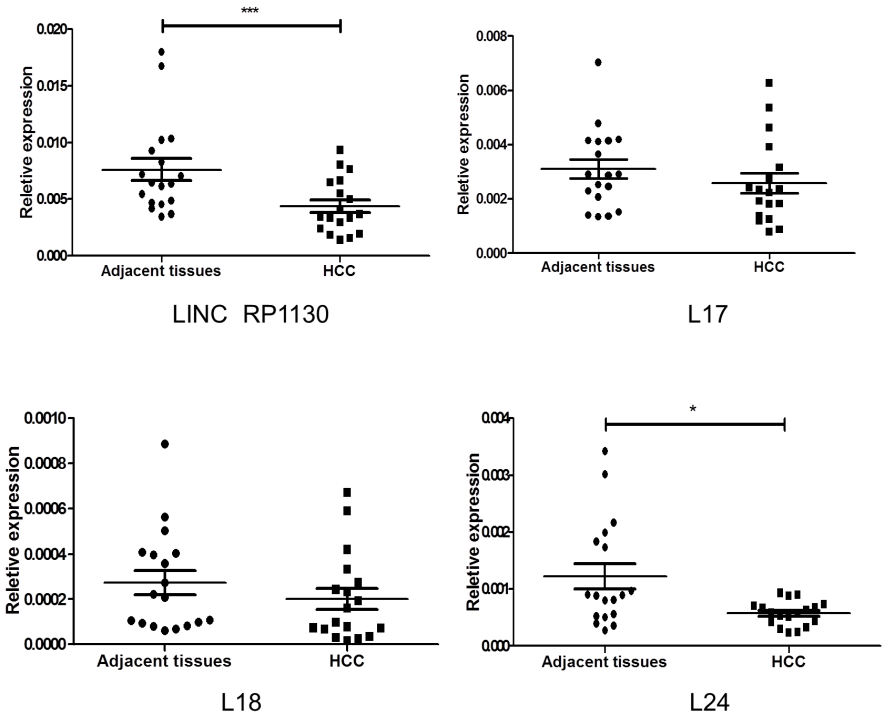


**Figure 2. Relative expression of 4 lncRNAs in 18 HCC tissues and paired adjacent non-cancerous hepatic tissues.** *: $p < 0.05$, ***: $p < 0.001$.

by a clinical pathologist. Tumor tissues and adjacent non-tumor tissue specimens were collected from the patients after obtaining informed consent, in accordance with the institutional guidelines of the hospital's Ethics Committee. Resected tumor tissue and adjacent normal tissue specimens were immediately snap-frozen in liquid nitrogen and stored in a tissue bank until use. The experimental operators were blinded to the clinical data. The human cell lines used in this study were obtained from the Experimental Research Support Center of Hospital 302 in Beijing (Beijing, China) and included HepG2, LO2, Huh7, and SMMC-7721 cells. All of the cell lines were maintained in an atmosphere of 5% $CO_2$ and grown in DMEM medium (Thermo, Beijing, China) supplemented with 10% fetal bovine serum (Gibco, Beijing, China).

### 2.2. *RNA preparation, reverse transcription, and quantitative real-time RT-PCR (qRT-PCR)*

Total RNA from frozen HCC tissues and adjacent non-tumor tissue samples ($n$ = 51) was extracted using TRIZOL reagent (Invitrogen Life Technologies, Carlsbad, CA, USA) according to the manufacturer's protocol. RNA integrity was evaluated using a NanoDrop ND-1000 spectrophotometer (NanoDrop Products, Wilmington, DE, USA), and cDNAs were synthesized from 50 ng of total RNA of each sample. Levels of LINC RP1130-1 expression were quantified with qRT-PCR performed on an ABI7500 system (Applied Biosystems, Foster City, CA, USA) using Maxima SYBR Green qRT-PCR master mix (Thermo Fisher Scientific, Waltham, MA, USA) according to the manufacturers' protocols. GAPDH expression was monitored as the endogenous control, and all samples were normalized to human GAPDH. All reactions were run in triplicate, using LINC RP1130-1-specific primers designed and synthesized by Sangon Biotech (Sangon, Shanghai, China). Their sequences were as follows: LINC RP1130-1 forward: 5'-ACCTCCCCACAAGCTGA-3', reverse: 5'- AACCGAATATTTGATGTCT-3'; GAPDH forward: 5'-CAGCCTCAAGATCATCAGCA-3' and reverse: 5'-TGTGGTCATGAGTCCTTCCA-3'. The amplification profile was 95˚C for 5 min, followed by 40 cycles of denaturation at 95˚C for 15 s, and annealing at 60˚C for 30 s. The median of three reactions was used to calculate relative lncRNA expression (ΔCt = Ct median lncRNA - Ct median GAPDH). Fold changes in expression were calculated using the $2^{-\Delta\Delta Ct}$ method.

### 2.3. *Statistical Analysis*

All experiments were performed in triplicate and repeated at least three times. Data are expressed as the mean ± S.D. Statistically significant differences between HCC tissues and adjacent non-tumor tissue samples were determined using the Wilcoxon signed-rank test, and differences between cell lines were determined using the Student's $t$ test. A receiver operating

characteristic (ROC) curve was plotted to determine how well the level of LINC RP1130-1 expression differentiated between HCC tissues and adjacent non-tumor tissues, and the cutoff value of 0.01 served as the level of expression sensitivity + specificity considered to be maximal. Associations between LINC RP1130-1 expression and clinicopathological characteristics were analyzed using a chi-squared test. Kaplan-Meier analyses were performed on the correlations between levels of LINC RP1130-1 expression and RFS. All statistical analyses were performed using SPSS for Windows software (ver. 16.0; SPSS Inc., Chicago, IL, USA). $p$ values < 0.05 were considered significant.

## 3. Results

### 3.1. *The level of LINC RP1130-1 expression decreased in HCC relative to that in adjacent non-tumor tissues*

To assess the potential clinical significance of LINC RP1130-1, its level of expression in both HCC tissues and adjacent non-tumor tissue specimens was analyzed with qRT-PCR. LINC RP1130-1 expression decreased significantly relative to that in adjacent non-tumor tissues ($p < 0.001$, Figure 3A, B, horizontal lines represent the median). LINC RP1130-1 expression was also examined with qRT-PCR in three HCC cell lines and the normal liver cell line. Results indicated that LINC RP1130-1 expression was lower in the HCC cell lines than in LO2 ($p < 0.05$, Figure 3C). ROC analysis was used to evaluate the ability of LINC RP1130-1 expression to differentiate between the tumor and control samples. The total area under the curve (AUC, representing accuracy of differentiation) was 0.74 for LINC RP1130-1 (Figure 3D), suggesting that the level of LINC RP1130-1 level has adequate sensitivity and specificity to differentiate between HCC tissues and adjacent non-tumor tissues.

### 3.2. *LINC RP1130-1 expression is correlated with clinical stage, the number of tumors, PVTT, liver cirrhosis, and microvascular invasion in patients with HCC*

To determine whether LINC RP1130-1 expression in HCC tissues is associated with clinicopathological parameters, the clinical stage, the number of tumors, and the presence of microvascular invasion were examined in samples from patients with HCC. As

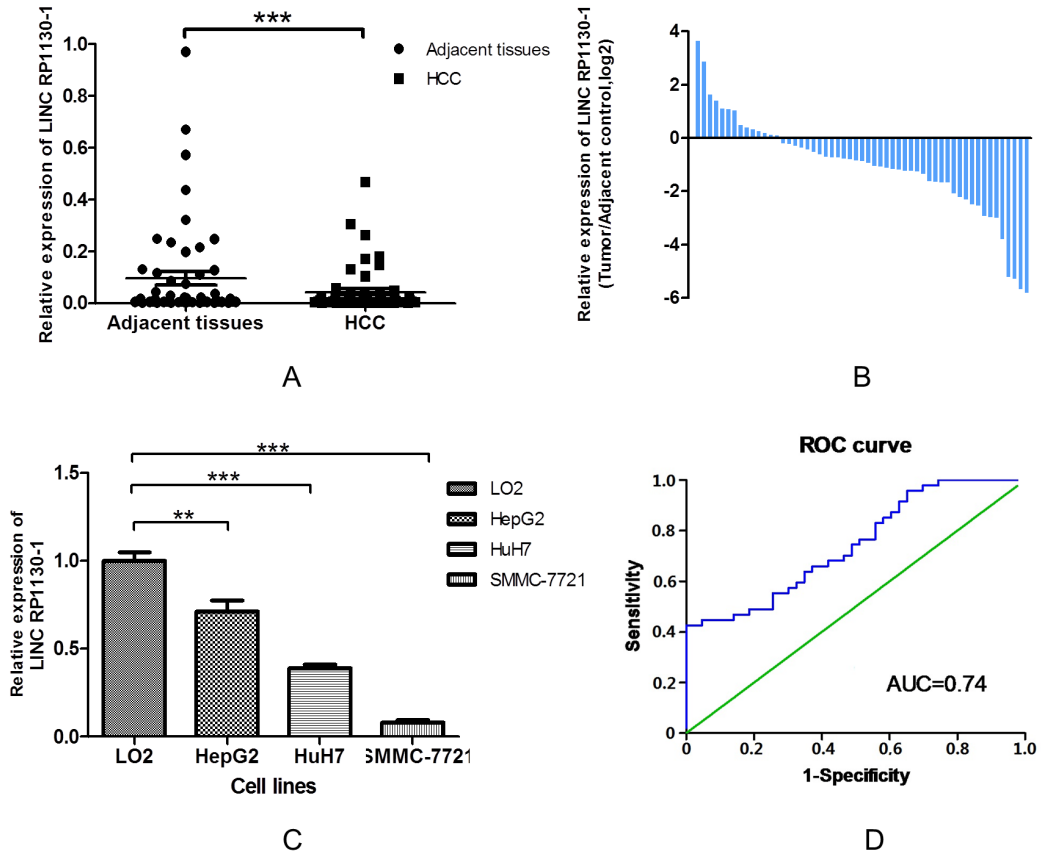


**Figure 3. Relative level of LINC RP1130-1 expression in patients with hepatocellular carcinoma (HCC). (A).** Relatively lower levels of LINC RP1130-1 expression were detected in HCC tissues than in adjacent non-tumor tissues from patients. **(B)**. Here, positive values for LINC RP1130-1 expression indicate a higher level of LINC RP1130-1 expression in tumor tissue than in non-tumor tissue and negative values indicate a lower level of LINC RP1130-1 expression in tumor tissue than in non-tumor tissue. **(C)**. The level of LINC RP1130-1 expression was lower in HCC cell lines than in LO2 ($p < 0.05$). **(D)**. The area under the receiver operating characteristic (ROC) curve was 0.74, distinguishing HCC from adjacent normal tissues. *: $p < 0.05$

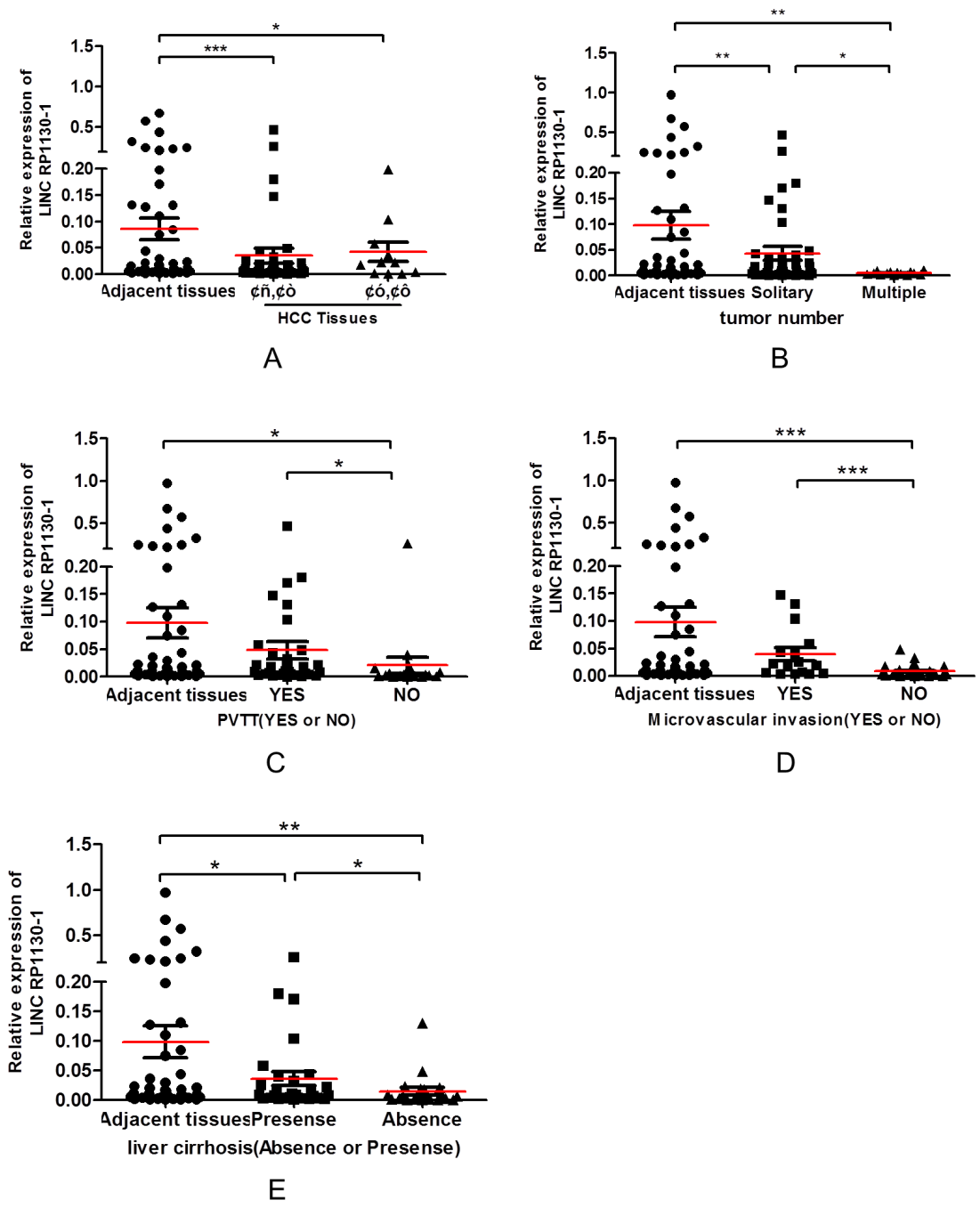


**Figure 4. LINC RP1130-1 expression is associated with clinical stage, the number of tumors, PVTT, microvascular invasion, and liver cirrhosis. (A)** LINC RP1130-1 expression in patients with stage I, II, III, or IV HCC was lower than in adjacent tissues. **(B)** LINC RP1130-1 expression was significantly higher in solitary HCC compared to multiple HCC. **(C)** LINC RP1130-1 expression changed markedly in patients with PVTT compared to patients without PVTT. **(D)** LINC RP1130-1 expression in patients with microvascular invasion differed significantly from that in patients without microvascular invasion. **(E)** Levels of LINC RP1130-1 expression in patients with liver cirrhosis were higher than those in patients without liver cirrhosis.

shown in Figure 4A, the level of LINC RP1130-1 expression was lower in tissue samples of clinical stage I and II HCC ($p < 0.001$) and clinical stage III and IV ($p < 0.05$) than in adjacent tissues. However, there was no significant difference in the level of expression in stage I, II, III, and IV. The level of LINC RP1130-1 expression was also lower in patients with multiple tumors than in those with solitary tumors (Figure 4B). In addition, the level of LINC RP1130-1 expression differed markedly in patients with PVTT or MVI than in those without PVTT or MVI (Figure 4C, D). The level of LINC RP1130-1 expression also varied significantly in patients with liver cirrhosis and those without liver cirrhosis (Figure 4E). The level of LINC RP1130-1 expression in patients with PVTT, MVI, or cirrhosis was higher than that in patients without PVTT, MVI, or cirrhosis, but that level was still lower than the average level of expression in adjacent tissues.

**Table 1. Association between LINC RP1130-1 and clinic pathological characteristics of patients with HCC[a]**

| Parameters | Total | LINC RP1130-1 expression | | *p* value |
|---|---|---|---|---|
| | | Low | High | |
| Gender | | | | |
| Male | 42 | 22 | 20 | 0.3 |
| Female | 9 | 3 | 6 | |
| Age (years) | | | | |
| < 60 | 36 | 19 | 17 | 0.406 |
| ≥ 60 | 15 | 6 | 9 | |
| Tumor size (cm) | | | | |
| <5 cm | 23 | 11 | 12 | 0.877 |
| ≥5 cm | 28 | 14 | 14 | |
| AFP | | | | |
| < 20 | 21 | 9 | 12 | 0.461 |
| ≥ 20 | 30 | 16 | 14 | |
| Histological grade | | | | |
| Well/moderate | 2 | 2 | 0 | 0.141 |
| Poorly | 49 | 23 | 26 | |
| Clinical stage | | | | |
| I and II | 40 | 23 | 17 | 0.021* |
| III and IV | 11 | 2 | 9 | |
| Number of tumors | | | | |
| Solitary | 44 | 24 | 20 | 0.048* |
| Multiple | 7 | 1 | 6 | |
| Alcohol consumption | | | | |
| Yes | 23 | 12 | 11 | 0.683 |
| No | 28 | 13 | 15 | |
| Smoking status | | | | |
| Yes | 19 | 9 | 10 | 0.856 |
| No | 32 | 16 | 16 | |
| HBV | | | | |
| Yes | 37 | 16 | 21 | 0.180 |
| No | 14 | 9 | 5 | |
| Recurrence | | | | |
| Yes | 20 | 9 | 11 | 0.644 |
| No | 29 | 15 | 14 | |
| PVTT | | | | |
| Yes | 33 | 12 | 21 | 0.014* |
| No | 18 | 13 | 5 | |
| Microvascular invasion | | | | |
| Yes | 19 | 18 | 1 | < 0.001*** |
| No | 32 | 7 | 25 | |
| Liver cirrhosis | | | | |
| Absence | 21 | 14 | 7 | 0.035* |
| Presence | 30 | 11 | 19 | |

[a]: Table 1 summarizes the association between LINC RP1130-1 expression and the clinic pathologic features of patients with HCC. A low level of LINC RP1130-1 expression was found to significantly correlate with clinical stage ($p = 0.021$), the number of tumors ($p = 0.048$), PVTT ($p = 0.014$), liver cirrhosis ($p = 0.035$), and microvascular invasion ($p < 0.001$). However, LINC RP1130-1 expression was not significantly related to gender, age, tumor size, histological grade, alcohol consumption, smoking status, HBV, or recurrence ($p > 0.05$). The median level of LINC RP1130-1 expression served as the cutoff. *: $p < 0.05$, ***: $p < 0.001$.

### 3.3. *Relationship between LINC RP1130-1 expression and RFS in patients with HCC*

Patients were divided into 2 groups, those with a level of LINC RP1130-1 expression below the 50th percentile who were classified as having lower LINC RP1130-1 levels ($n = 26$). Patients with a level of LINC RP1130-1 expression above the 50th percentile were classified as having higher LINC RP1130-1 levels ($n = 25$). A second analysis yielded similar results, *i.e.* the level of LINC RP1130-1 expression was related to clinical stage, multiple tumors, and the presence of PVTT, MVI, or liver cirrhosis, but there was no significant correlation between LINC RP1130-1 expression and other clinicopathological features, including age, gender, tumor size, histological grade, alcohol consumption, smoking status, hepatitis B virus (HBV), and recurrence (Table 1). However, Kaplan-Meier analysis of the relationship between the level of LINC RP1130-1 expression and RFS indicated that patients with lower levels of LINC RP1130-1 expression had a significantly shorter RFS than patients with higher levels of expression ($p < 0.05$) (Figure 5).

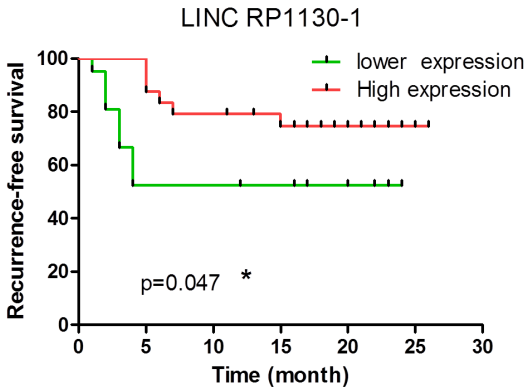


**Figure 5. Kaplan-Meier curves for recurrence-free survival (RFS) in patients with HCC and a low or high level of LINC RP1130-1 expression**

## 4. Discussion

This study noted dys-regulation of LINC RP1130-1 in HCC tissues compared to matching adjacent non-tumor tissues. Results indicated that LINC RP1130-1 expression in HCC decreased significantly in both stage I and II ($p < 0.0001$) and in stage III and IV ($p < 0.05$). The AUC was 0.74 for LINC RP1130-1, indicating its specificity and sensitivity in the diagnosis of HCC, and patients with HCC and low levels of LINC RP1130-1 expression had a significantly shorter RFS ($p = 0.047$) than did patients with high levels of expression. Expression of LINC RP1130-1 differed significantly depending on the presence of PVTT or cirrhosis ($p < 0.05$), the number of tumors ($p < 0.05$), and microvascular invasion ($p < 0.001$). This suggests the prognostic value of LINC RP1130-1. Overall, LINC RP1130-1 may play an important role in the development and progression of HCC.

Numerous studies have found that factors, including the stage of HCC, the presence of PVTT or cirrhosis, and the number of tumors, have considerable significance in tumor recurrence. MVI is a sign of the invasive nature of HCC and is the most important predictor of HCC recurrence after surgery. Angiogenesis in tumor tissues

can lead to microvascular thrombus formation, which is a critical risk factor associated with intrahepatic metastasis of HCC. Recent studies have found that MVI is an independent risk factor for HCC recurrence and that microvascular thrombi tend to express different levels of lncRNA compared to tumor tissues. MVI has clinical significance in monitoring recurrence and guiding adjuvant therapy. Microvascular thrombi were difficult to detect in imaging tests and were always confirmed *via* a pathological examination after surgery. Thus, identifying a biomarker of MVI would greatly facilitate prediction of HCC recurrence. However, the current data indicated that levels of LINC RP1130-1 expression in patients without PVTT or MVI were lower than those in patients with PVTT or MVI, which was contrary to expectations. Samples were selected randomly instead of pairing patients with certain clinical indicators, which may have caused an uneven distribution of samples with specific characteristics and thus account for this unexpected result. As an example, only 7 patients had multiple tumors while the rest had solitary tumors. That said, the extent of intratumor heterogeneity may also account for this result. A recent study has shown that different subtypes of tumor cells with unique expression profiles were distributed within the same tumor lesions and that this distribution may change depending on the progression of tumor (*35*). The size of the clinical sample should be increased in subsequent experiments in order to verify the level of LINC RP1130-1 expression, especially in patients with PVTT or MVI. However, the level of LINC RP1130-1 expression consistently decreased in HCC, and it still should be able to serve as a potential biomarker of HCC.

HCC develops frequently when fibrosis is in an advanced stage, so eradicating HBV or HCV infection is a promising prophylactic therapy to prevent the occurrence of liver fibrosis and HCC (*36*). Numerous lncRNAs have been found to participate in the development and progression of liver cancer, but few studies have reported on the role of lncRNAs in the process of cirrhosis. Human hepatic stellate cells (HSCs) are closely related to cirrhosis. A recent study has reported that more than 3,600 lncRNAs are expressed at different levels in HSCs and that 400 lncRNAs are specifically expressed in HSC (*37*), suggesting that lncRNAs may play a key role in the progression of cirrhosis. The lncRNA HULC was found to be up-regulated in plasma samples from patients with HBV-related cirrhosis (*38*). The current study indicated that LINC RP-1130 tends to be expressed at lower levels in patients with cirrhosis than patients without cirrhosis, suggesting that some LncRNA expression profiles may change in patients with HCC and cirrhosis.

In conclusion, the current results are the first to indicate that LINC RP1130-1 levels were significantly lower in HCC tissues and that dysregulation of LINC RP1130-1 was correlated with PVTT and MVI in patients with HCC. Patients with HCC and low levels of LINC RP1130-1 expression had a significantly shorter RFS than did patients with high levels of expression. These findings indicate that LINC RP1130-1 may have potential as a diagnostic and prognostic biomarker for HCC.

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*(Received July 3, 2016; Revised October 12, 2016; Accepted October 13, 2016)*