Upregulation of Long Non-Coding RNA PlncRNA-1 Promotes Metastasis and Induces Epithelial-Mesenchymal Transition in Hepatocellular Carcinoma

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Key Words
PlncRNA-1 • Hepatocellular carcinoma • Oncogene • Proliferation • Invasion

Abstract

Background/Aims: PlncRNA-1 has been demonstrated to promote malignancy in various cancers. The present study aims to investigate the expression pattern, prognosis value and the function of PlncRNA-1 in human hepatocellular carcinoma (HCC).

Methods: The expression of PlncRNA-1 in 84 pairs of HCC and their matched normal tissues was examined by quantitative real-time polymerase chain reaction (qRT-PCR). The correlations of PlncRNA-1 expression and clinicopathological characteristics and prognosis were also analyzed. The biological role of PlncRNA-1 in cell proliferation, migration and invasion was examined in vitro and in vivo.

Results: The results showed that the level of PlncRNA-1 expression was significantly increased in HCC tissues and significantly correlated with tumor size, vascular invasion and advanced TNM stage. Moreover, patients with high levels of PlncRNA-1 expression had relatively poor prognostic outcomes, serving as an independent prognostic factor for HCC. In vitro functional assays indicated that knockdown of PlncRNA-1 expression significantly reduced cell proliferation, migration and invasion by inhibiting the epithelial-mesenchymal transition (EMT) signaling. Animal model experiments confirmed the ability of PlncRNA-1 to promote tumor growth in vivo.

Conclusions: Taken together, our findings suggest that PlncRNA-1 may serve as an oncogene in HCC progression and represent a valuable prognostic marker and potential therapeutic target for HCC.
poor prognosis of HCC [2, 3]. It is well known that early metastasis has tight association with epithelial-to-mesenchymal transition (EMT) in cancer cells, which accelerates tumor migration and invasion [4]. Therefore, better understanding of the molecular mechanisms involved in HCC invasion and metastasis is essential for the improvement of diagnosis, therapy and prognosis prediction of HCC. Recently, accumulating evidence indicate that long noncoding RNAs (lncRNAs) play a crucial role in HCC pathogenesis, providing new insights into the biology of this disease [5].

lncRNAs are transcripts longer than 200-nucleotide RNAs with no or little protein coding function and participate in a large range of biological processes, including regulating gene expression through modulation of chromatin remodeling, controlling gene transcription, post-transcriptional mRNA processing, protein function or localization, and intercellular signaling [6, 7]. To date, thousands of lncRNAs have been discovered via chromatin signature analysis and large-scale sequencing [8]. Their dysregulation has been found in various types of malignancies [9, 10], and abnormal expressions of certain lncRNAs are associated with tumor growth, carcinogenesis, or metastasis [11-13]. However, the biological function and clinical significance of lncRNAs in cancer remain largely unknown.

PlncRNA-1 (prostate cancer-up-regulated long noncoding RNA 1, also known as CBR3-AS1), a new lncRNA which locates in the antisense region of carbonyl reductase 3 (CBR3), was firstly found to be generally overexpressed in prostate cancer (CaP) cell lines and tissues [14]. Knockdown of PlncRNA-1 expression resulted in defects in cell proliferation and increased rates of apoptosis in CaP cells through reciprocal regulation of androgen receptor [14]. Additional study reported that upregulation of PlncRNA-1 promotes cell proliferation and correlates with advanced tumor stage and lymph node metastasis in esophageal squamous cell carcinoma (ESCC) [15]. However, to the best of our knowledge, the biological role and clinical significance of PlncRNA-1 in HCC development and progression remains poorly understood.

In this study, we found that the expression of PlncRNA-1 was significantly upregulated in HCC tissues compared with adjacent normal tissues. Its expression level was significantly correlated with tumor size, vascular invasion and advanced TNM stage. Moreover, high PlncRNA-1 expression correlated with poor patient prognosis. Furthermore, in vitro functional assays showed that knockdown of PlncRNA-1 expression significantly reduced cell proliferation, migration and invasion by inhibiting the epithelial-mesenchymal transition (EMT). Moreover, using an in vivo animal model, we demonstrated that PlncRNA-1 plays an oncogenic role in HCC by promoting tumor growth. Our findings may provide the better understanding on the roles and its clinic implications of PlncRNA-1 in the development and progression of HCC.

Materials and Methods

Patients and tissue specimens

A total of 84 paired our and matched normal adjacent liver tissues were collected from HCC patients underwent resection at The First Affiliated Hospital of Wenzhou Medical University between 2005 and 2009. Tumor stages were determined by TNM classification according to the 2002 International Union Against Cancer guidelines. Tumor differentiation was graded according to Edmondson-Steiner classification. Clinical characteristics of all patients are given in Table 1. Data were censored at the last follow-up for patients without recurrence or death. Overall survival (OS) time was defined as the interval from primary surgical treatment to the first recurrence or death, respectively. The study was approved by the Ethics Committee of The First Affiliated Hospital of Wenzhou Medical University, and informed consent was obtained from each patient according to the committee’s regulations.

Mice, cell lines, antibodies and siRNA transfection

Male 6-8 weeks old BALB/c nu/nu mice (Shanghai Institute of Material Medicine, Chinese Academy of Science) were housed in specific pathogen-free conditions. All animals received humane care according
to the criteria outlined in the "Guide for the Care and Use of Laboratory Animals" prepared by the National Academy of Sciences and published by the National Institutes of Health (NIH publication 86-23 revised 1985).

Four human HCC cell lines, including HCCLM3, Huh7, SK-Hep1, HepG2, and one normal liver cell line L02 were used in this study. These cell lines were all purchased from American Type Culture Collection (ATCC, Rockville, MD, USA) and were cultured in Dulbecco's modified Eagle's medium (Gibco, Grand Island, NY, USA) supplemented with 10% fetal bovine serum and maintained at 37°C in a humidified incubator containing 5% CO₂. Polyclonal antibodies against E-cadherin, N-cadherin, vimentin, snail, β-actin and secondary antibodies were all purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA).

The sequences of PlncRNA-1 target-specific siRNA duplexes and control siRNA were designed as previously described [14]. All siRNAs were chemically synthesized by Shanghai GenePharma Company (Shanghai, China). For siRNA transfection, HCCLM3 and SK-Hep1 cells were seeded in a 6-well culture plate at a density of 3 × 10⁵ cells/well and transfected with 50 nM PlncRNA-1-targeting siRNA using Lipofectamine® 2000 (Invitrogen, Life Technologies, Carlsbad, CA, USA) according to the manufacturer’s instructions. Non-targeting siRNA was used as the control. Forty-eight hours after transfection, the cells were harvested for further studies. The transfection efficiency was determined by RT-quantitative PCR.

RNA extraction and real-time quantitative PCR
Total RNA was extracted from HCC tissues, cell lines or human hepatocyte with TRIzol (Invitrogen, Carlsbad, CA, USA) according to the manufacturer’s protocol. Total RNA concentration was assessed by measuring absorbance at 260 nm using a NanoDrop spectrophotometer (ND-1000, Thermo Scientific, Waltham, MA, USA). Quality of RNA was generally checked by the ratios of A260/A280 and A260/A230 and RNA integrity was assessed by electrophoresis through denaturing agarose gels. 2μg of total RNA was reversely transcribed using M-MLV reverse transcriptase (Life Technologies Inc., Gaithersburg, MD, USA) according to the manufacturer’s recommendations. The relative expression levels of PlncRNA-1 were determined by real-time quantitative PCR. Gene-specific amplification was performed using ABI 7500 fast real-time PCR system (Applied Biosystems, Foster City, CA, USA) and SYBR Green PCR Master Mix (Applied Biosystems, Foster City, CA, USA) according to the manufacturer’s instructions. The following gene-specific primers were used in this study: forward, 5'-GCG GGA GTC TTC CTT AGCTT-3' and reverse 5'-GGT CAC GGT CTT ATC GAGGA-3' for PlncRNA-1; forward, 5'-CTG TCC TCG CCG TCA CACCG-3' and reverse, 5'-GGC ATG GAC TGT GGT CAT GAG-3' for GAPDH. Relative gene expression was quantified using the comparative delta CT (2⁻ΔΔCT) method, as previously described [15].

Human hepatocyte isolation
Human hepatocytes were isolated from liver biopsy specimens using a modified ‘two-step’ collagenase perfusion procedure as previously described [16]. In short, liver biopsy specimens were washed, perfused, and digested in Hank’s Balanced Salt Solution (HBSS) supplemented with 0.5% collagenase A (Sigma-Aldrich), 0.25% Protease (Type XIV, Sigma), 0.125% 0.002% Hyaluronidase (Sigma), and 0.05% DNase I (Roche) at 37°C for 20 minutes. Dissociated cells were filtered through a 150-μm mesh and separated by Percoll density gradient centrifugation at 300×g for 30 min at room temperature. Cell viability was determined by trypan blue dye exclusion assay.

Cell proliferation assay
Cell growth was determined using CCK-8 (Beyotime Biotechnology, Haimen, China) assay according to manufacturer’s instructions. Briefly, HCCLM3 and SK-Hep1 cells were transfected with 50 nM si-PlncRNA-1 or si-NC. After 24 h, the transfected cells were harvested, seeded into 96-well plates at 2×10⁴ cells per well and cultured for 1, 2, 3, 4, days before addition of 10 μl CCK-8 (5 mg/ml) to the culture medium in each well. After 1 hour incubation at 37°C, OD values were read using a microplate reader (Bio-Tek Company, Winooski, VT, USA) at the 450-nm wavelength. Each time point was repeated in three wells and the experiment was independently performed for three times.

Cell migration and invasion assay
Cell migration and invasion abilities were assessed using in vitro Boyden chamber assay with 24-well transwells (8-μm pore size; Minipore, Billerica, MA, USA). For the migration assay, 1 × 10⁴ transfected
cells were suspended in 200 μL Dulbecco’s modified Eagle medium with 1% fetal bovine serum and were added to the upper chamber. For the invasion assays, the membrane was precoated with 45 μg Matrigel (BD Biosciences, Franklin Lakes, NJ, USA) to form a matrix barrier. 600 μL Dulbecco’s modified Eagle medium with 10% fetal bovine serum was placed in the lower chamber. After 24 hours of incubation, the cells remaining on the upper membrane were removed with cotton wool. Cells that had migrated or invaded through the membrane were fixed in 4% paraformaldehyde and stained with 0.5% crystal violet (Beyotime Institute of Biotechnology, Shanghai, China) for 30 min, rinsed in phosphate-buffered saline, and cells in five microscopic fields (at 200× magnification) were counted and photographed. Experiments were independently repeated three times.

In Vivo tumor growth assay

1 × 10^6 HCCLM3 cells were injected subcutaneous into the flank region of 6-8 week-old female nude mice. The mice were divided into two treatment groups of ten animals: siPlncRNA-1 and siRNA-NC. Entranster-in vivo transfection reagent was purchased from Engreen Biosystem (Beijing, China). The Entranster-in vivo-siRNA mixture was prepared according to the manufacturer’s instructions. The method performed has been described previously [17, 18]. Briefly, 5 μg of PlncRNA-1-siRNA or control siRNA (siRNA-NC) was dissolved in 5 μL of RNase-free water. Next, 5 μL of siRNA solution was mixed with 5 μL of the Entranster-in vivo transfection reagent. The mixture was locally injected into the tumor mass every 3 days for 3 weeks. Tumor growth was monitored every 3 days and tumors were measured with fine digital calipers and tumor volume was calculated by the following formula: tumor volume = 0.5 × width^2 × length.

Western Blotting

20 μg of total protein form xenografts tissues was extracted and separated by 10% SDS-PAGE, after which the protein was transferred onto polyvinylidene fluoride membranes, and then the membranes were incubated with anti-human E-cadherin, N-cadherin, vimentin and β-actin antibodies (Santa Cruz Biotech, Santa Cruz, CA, USA) at 4°C overnight. The membranes were then washed three times with TBST (tris-buffered saline with tween-20) and probed with the horseradish peroxidase (HRP)-conjugated anti-IgG antibody at room temperature for 2 hour. The bands were visualized using BioImaging Systems (UVP Inc., Upland, CA, USA).

Statistical analysis

Statistical Package of the Social Sciences 16.0 for Windows (SPSS, Chicago, IL, USA) was used for statistical analyses. Differences in PlncRNA-1 expression were evaluated with the paired-samples t-test or Mann-Whitney U test. The relationships between PlncRNA-1 expression and various clinicopathological parameters were analyzed by chi-square test. Survival curves were plotted by the Kaplan-Meier method and compared using the log-rank test. The Cox proportional hazard regression model was used for univariate and multivariate analyses to study the effects of the clinicopathological variables and PlncRNA-1 expression on survival. Differences in cell growth and cell invasion were assessed using the two-tailed unpaired Student’s t test. P < 0.05 was considered as statistical significance.

Results

PlncRNA-1 expression is up-regulated in human HCC tissues and correlated with tumor stage and metastasis

We first determined PlncRNA-1 mRNA expression in 84 paired tumor and matched adjacent normal liver tissues via real-time quantitative RT-PCR. The relative expression of PlncRNA-1 was significantly upregulated in HCC tissues compared with the adjacent normal tissues (70 of 84 cases (83.3%), P < 0.001, Fig. 1a). Furthermore, comparisons of the clinical pathological variables with PlncRNA-1 expression in HCC were made. PlncRNA-1 expression levels less than the mean expression level (2^ΔΔCt = 10.41) were assigned to the low expression group (n = 36), and those samples with expression above the mean value were assigned to the high expression group (n = 48). Correlation analysis of PlncRNA-1 expression with clinical pathological features of HCC patients revealed a significant positively association between increased PlncRNA-1 expression and tumor size, vascular invasion and advanced
TNM stage (Fig. 1b-d). However, PlncRNA-1 expression was not correlated with other or pathological parameters, including age, gender, hepatitis history, liver cirrhosis history, AFP level, ALT level, tumor multiplicity and tumor differentiation (Table 1).

**Upregulation of PlncRNA-1 is associated with a poor prognosis of HCC**

The correlation between PlncRNA-1 expression and HCC patient prognosis was evaluated by Kaplan-Meier survival analysis and log-rank tests. The 5-year overall survival (OS) rates in patients with high and low PlncRNA-1 expression were 24.5% and 52.5%, respectively (Fig. 2a, log rank, \( P < 0.001 \)). The median survival time for low PlncRNA-1 expression is 37.8 months while for high PlncRNA-1 expression 25.4 months.

Univariate analyses showed that hepatitis history, AFP level, tumor size, vascular invasion, TNM stage and PlncRNA-1 expression were significantly associated with OS (Table 2). Furthermore, the multivariate Cox regression analysis confirmed the tumor size, vascular invasion, TNM stage and PlncRNA-1 expression were independent prognostic factors for OS of HCC patients (Table 2). These results suggest that PlncRNA-1 upregulation plays a critical role in HCC development and progression.

**PlncRNA-1 Expression is up-regulated in HCC Cell Lines**

We next performed RT-qPCR analysis to examine the expression of PlncRNA-1 in four human HCC cell lines and one normal liver cell line (L02 cells). The results showed that the expression of PlncRNA-1 was higher in all four HCC cell lines compared with the levels
observed in L02 cells and normal hepatocytes (Fig. 2b).

**Down-regulation of PlncRNA-1 reduces HCC cell proliferation and invasion in vitro**

Based on the findings mentioned above, we hypothesized that PlncRNA-1 was implicated in HCC growth and metastasis. To investigate the biological function of PlncRNA-1 in HCC, RNAi was employed to knock down endogenous PlncRNA-1 in 2 HCC cell lines HCCLM3 and SK-Hep1. As shown in Fig. 2c, the RT-qPCR data confirmed the PlncRNA-1 level was significantly reduced in HCCLM3 and SK-Hep1 cells than their siRNA control cells.

Using these transient transfection cell lines, we next evaluated the role of PlncRNA-1 on cell proliferation, migration, and invasion. As shown in Fig. 3a and b, time course CCK-8 assay revealed that, forty eight hours after seeding the cells, the cell growth was significantly inhibited in the PlncRNA-1 knockdown HCC cells compared with control cells. Furthermore, *In vitro* migration and invasion assay showed that downregulation of PlncRNA-1 decreased their migration and invasion capabilities by approximately 60% and 40%, respectively (Fig. 3c and d). These results suggest that PlncRNA-1 plays an oncogenic role in promoting the progression and metastasis of HCC.

**Table 1. Correlation between PlncRNA-1 expression and clinicopathological characteristics of HCC patients**

<table>
<thead>
<tr>
<th>Characteristics</th>
<th>Cases (84)</th>
<th>PlncRNA-1 expression</th>
<th>P value</th>
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<td>Age (years)</td>
<td></td>
<td></td>
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<td>≤55</td>
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<tr>
<td>&gt;55</td>
<td>24</td>
<td>Low (36)</td>
<td>10 (27.8%)</td>
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<tr>
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<td></td>
<td>38 (79.2%)</td>
</tr>
<tr>
<td>Female</td>
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<td></td>
<td>10 (20.8%)</td>
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<tr>
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<td></td>
<td>35 (72.2%)</td>
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<tr>
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<tr>
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</table>

**Down-regulation of PlncRNA-1 suppresses tumor growth in vivo**

To further address the tumor-promoting function of PlncRNA-1, we performed an *in vivo* xenograft model. HCCLM3 cells were injected subcutaneous into the flank region of nude mice. siPlncRNA-1 and siRNA-NC were mixed with *in vivo* transfection reagent, and locally injected into the tumor mass every 3 days for 3 weeks. After 21 days of growth, the tumor volume and weight was reduced in approximately 67% of mice harboring grafts knockdown of PlncRNA-1 compared with that in the mice of the siRNA control (Fig. 4a-c). RT-qPCR analysis of tumor tissues showed that the mRNA expression of PlncRNA-1 was significantly decreased after knockdown of PlncRNA-1 (Fig. 4d).
PlncRNA-1 promotes HCC metastasis by regulating EMT signaling

Accumulating evidence shows that EMT is involved in the progression and metastasis of various cancers. To investigate the potential mechanism by which PlncRNA-1 affected cell
metastasis and invasion of HCC. The expression of the epithelial markers, E-cadherin, and the mesenchymal markers, N-cadherin and Vimentin, was examined. As shown in Fig. 4e and f, the expression of E-cadherin was upregulated in PlncRNA-1 knockdown HCCLM3 cells compared with that in control cells, whereas the N-cadherin and Vimentin expressions were significantly decreased in PlncRNA-1 knockdown HCCLM3 cells. Additionally, we found that knockdown of PlncRNA-1 in xenografts tissues by siPlncRNA-1 resulted in upregulation of E-cadherin and decreased of N-cadherin and Vimentin (Fig. 4g and h). These results suggest that PlncRNA-1 promotes HCC progression by regulating the EMT signaling.

Fig. 3. PlncRNA-1 suppresses the proliferation, migration and invasion of HCC cells in vitro. HCCLM3 and SK-Hep1 cells were transfected with si-PlncRNA-1 or siRNA-NC, respectively. (a and b) Cell proliferation was analyzed by time course CCK-8 assay. (c and d) Cell migration and invasion ability were performed by Transwell or Matrigel precoated Transwell assay. Data shown are mean ± SD from three independent experiments. *P < 0.05, **P < 0.01, ***P < 0.001.
Discussion

Increasing evidence suggests that lncRNA dysregulation has been found in various types of malignancies, and their differential expressions are closely related to tumorigenesis, metastasis and prognosis [11]. lncRNA PlncRNA-1 (NCBI no. BC038671) is a new lncRNA transcript which is located in the antisense region of carbonyl reductase 3 (CBR3) that encodes a protein belonging to the shortchain dehydrogenases/reductases family [19]. Several groups have reported that PlncRNA-1 is frequently upregulated in human cancers, including CaP and ESCC [14, 15]. However, up to now, there is no relevant report about the relationship between PlncRNA-1 and the progression of HCC. Thus, the role of PlncRNA-1 in HCC and its underlying mechanism remain to be determined.
In the present study, by using quantitative RT-PCR, we found that the expression of PlncRNA-1 was upregulated in HCC tissues and cell lines compared with normal tissues as well as normal liver cells. Notably, PlncRNA-1 expression was found to be significantly higher in patients with advanced tumor stage and in patients that had undergone vascular invasion. Additionally, the overall survival time of patients with higher PlncRNA-1 expression levels was shorter than that of patients with lower PlncRNA-1 expression levels. PlncRNA-1 expression was an independent prognostic factor for OS of HCC patients. Cui et al. first reported that the expression of PlncRNA-1 was significantly higher in CaP cells relative to normal prostate epithelial cells, as well as higher in human CaPs compared with normal tissues and benign prostatic hyperplasia (BPH) [14]. Wang et al. found that the expression of PlncRNA-1 was significantly higher in human ESCC compared with the adjacent noncancerous tissues, and the high level of PlncRNA-1 expression was significantly correlated with advanced clinical stage and lymph node metastasis [15]. Taken together, our observation in current study is in agreement with previous studies revealing that PlncRNA-1 was an independent prognostic factor for HCC and disease progression.

To further investigated the biological role of PlncRNA-1 in HCC. We employed siRNA approach to knockdown PlncRNA-1 expression in two HCC cell lines HCCLM3 and SK-Hep1 with high endogenous PlncRNA-1 level. RNAi-mediated suppression of PlncRNA-1 in HCCLM3 and SK-Hep1 cells led to a significant inhibition cell proliferation, migration and invasion in vitro as well as tumor growth in vivo. This is the first report to demonstrate the functional role of PlncRNA-1 in human HCC in vitro and in vivo. Consistent with our findings, previous studies have identified that PlncRNA-1 has regulating role in modulating cell proliferation and apoptosis. In a previous study of CaP, Silencing of PlncRNA-1 significantly reduced cell proliferation and induced apoptosis in CaP cell lines [14]. In a further study, knockdown of PlncRNA-1 reduced ESCC cell colony formation, proliferation, cycle transit from G1 to S phase and increased apoptosis [15]. These data, together with ours, indicate that PlncRNA-1 functions as an oncogene and promotes HCC malignant progression, PlncRNA-1 may represent a novel therapeutic target for HCC treatment.

The molecular mechanisms by which PlncRNA-1 inhibits cancer cell proliferation and metastasis remain unclear. Previous studies identified a significant correlation between PlncRNA-1 and androgen receptor signaling pathway in CaP [14]. Accumulating evidence shows that EMT is associated with tumor metastasis, invasiveness and prognosis [20]. Moreover, numerous studies have identified functional correlation between IncRNAs and EMT during carcinogenesis, including IncRNA-H1T [21], Linc00152 [22], SPRY4-IT1 [23] and AOC4P [24]. Therefore, in the present study, we examined the expressions of EMT related protein E-cadherin, N-cadherin, fibronectin and Vimentin. We found that knockdown of PlncRNA-1 in HCCLM3 cells led to a decreased expression of the epithelial marker, E-cadherin, and increased expression of mesenchymal markers, N-cadherin, Vimentin and fibronectin. These results suggest that PlncRNA-1 promotes HCC invasion by inducing EMT.

In conclusion, our study for the first time demonstrated that PlncRNA-1 was upregulated in HCC and its upregualtion was correlated with advanced TNM stage, vascular invasion, and poor overall survival of HCC patients. In addition, we certified that PlncRNA-1 can inhibit HCC cell proliferation, migration and invasion in vitro and in vivo, partly through regulation of EMT signaling. Given these findings, our data demonstrate that PlncRNA-1 may serve as an oncogene in HCC progression and represent a valuable prognostic marker and potential therapeutic target for HCC, further studies are needed to investigate its molecular mechanisms.

**Disclosure Statement**

The authors declare that they have no competing interests.
References