Downregulation of long noncoding RNA TINCR inhibits cell proliferation, invasion, and epithelial-mesenchymal transition of hepatocellular carcinoma

Running title: Downregulation of long noncoding RNA TINCR inhibits HCC progression

Hongjuan Li[1&], Yaqin Chen[2&], Chunyan Wu[3], Haiyan Zhao[4], Xuesong Zhang[5*], Caidi Zhu[6*]

1.Department of Oncology, Jingjiang People's Hospital, Jingjiang, Jiangsu 214500, P. R. China.

2.Department of Infectious, Jingjiang People's Hospital, Jingjiang, Jiangsu 214500, P. R. China.

3.Department of Nursing, Jingjiang People's Hospital, Jingjiang, Jiangsu 214500, P. R. China.

4.Department of Digestive, Jingjiang People's Hospital, Jingjiang, Jiangsu 214500, P. R. China.

5.Department of Central laboratory, Jingjiang People's Hospital, Jingjiang, Jiangsu 214500, P. R. China.

6.Department of geriatric medicine, Jingjiang People's Hospital, Jingjiang, Jiangsu 214500, P. R. China.

& Co-first Author

*Corresponding to: Xuesong Zhang, Email: Y857da2@tom.com, Department of Central laboratory, Jingjiang People's Hospital, 28 Zhongzhou Road,Jingjiang, Jiangsu 214500, P. R. China.

Caidi Zhu, Email: caizhu023@outlook.com, Department of geriatric medicine, Jingjiang People's Hospital, 28 Zhongzhou Road, Jingjiang, Jiangsu 214500, P. R. China.

Abstract

Accumulating reports have identified that long non-coding RNAs (lncRNAs) function as key regulators of tumor initiation and progression. The aim of the current study was to determine the clinical significance and functional role of TINCR in hepatocellular carcinoma (HCC). In the present study, the level of lncRNA TINCR expression was significantly upregulated in HCC tissues compared to adjacent normal tissues. Higher levels of lncRNA TINCR expression

were significantly correlated with tumor size and vascular invasion of HCC patients. LncRNA TINCR knockdown inhibited cell proliferation ability, increased the proportion of G1 phase cells, reduced the proportion of S phase cells, and suppressed cell invasion of HCC *in vitro*. Additionally, lncRNA TINCR knockdown inhibited the HCC cell epithelial-mesenchymal transition (EMT) phenomenon by upregulating E-cadherin and reducing N-cadherin expression. We demonstrated that knockdown of lncRNA reduced tumor growth *in vivo*. Thus, these results indicated that lncRNA TINCR exhibits a tumor oncogenic role in HCC and inhibition of lncRNA TINCR might serve as a therapeutic target for HCC.

Keywords: Long non-coding RNA, hepatocellular carcinoma, TINCR, cell proliferation, cell invasion

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Introduction

Hepatocellular carcinoma (HCC), ranking as the fifth most common cancer worldwide, has extremely high morbidity and mortality rates [1]. In 2012, > 700,000 people died of HCC, which accounts for 9.1% of all cancer-related deaths [2]. The poor survival rate for advanced staged patients results from tumor recurrence and metastasis [3]. Therefore, identifying novel biomarkers for early stage detection and a novel potential therapeutic target of HCC is desirable and urgently needed.

Long non-coding RNAs (lncRNAs) have emerged as key regulators in tumor proliferation, migration, invasion, and metastasis, including HCC [4]. Recent studies have demonstrated some lncRNAs are abnormally expressed and involved in the pathogenesis and metastasis of HCC [5]. HULC [6], NEAT1 [7], CCAT1 [8], and AFAP1-AS1 [9] act as oncogenes, while others lncRNAs, including GAS5 [10] and MEG3 [11], function as tumor suppressors in HCC. TINCR has been reported to have higher expression in HCC and is associated with an unfavorable prognosis in patients with HCC [12]. The underlying functional role of TINCR in HCC, however, has not been established.

In the current study we found that the level of lncRNA TINCR expression was significantly upregulated in HCC tissues and correlated with tumor size and vascular invasion. Furthermore, lncRNA TINCR knockdown inhibited cell proliferation *in vitro* and *in vivo*. Additionally, lncRNA TINCR knockdown inhibited HCC cell invasion and epithelial-mesenchymal transition (EMT). These results indicated that

lncRNA TINCR has a tumor oncogenic role in HCC and inhibition of lncRNA TINCR might serve as a therapeutic target for HCC.

Methods

Patient tissue specimens

63 HCC tissues specimens and adjacent normal tissues specimens from patients were obtained at Jingjiang People's Hospital from July 2018 to April 2020. Human HCC tissues specimens were diagnosed by professional pathologists. After operation, the HCC tissues specimens and adjacent normal tissues specimens were immediately placed in liquid nitrogen for RNA analysis. No patients received therapies including radiotherapy, chemotherapy and radiofrequency ablation prior to surgery. Tumor staging was determined by the seventh edition of staging system of American Joint Committee on Cancer (AJCC) issued in 2010. The study was approved by Ethic Committee of Jingjiang People's Hospital and written informed consent was obtained from all of patients.

RNA extraction and qRT-PCR

Total RNA from HCC tissues and cells was extracted by Trizol reagent (TAKALA, Dalian, China) according to the manufacturer's instruction. The cDNA templates were constructed from RNA via Super M-MLV reverse transcriptase Kit (TAKALA, Dalian, China). QRT-PCR assay was performed by using FastStart Universal SYBR Green Master (TAKALA, Dalian, China) on ABI 7500 system (Applied Biosystems, Foster City, CA, USA). The mRNA expression was normalized to GAPDH. The primer sequences were below: TINCR forward: 5'-CCTTCCCATCTGTTCTCCCTTCC-3',TINCRreverse:5'-CTGTATCTAGTTCC AAGCTGGGTGAT-3'; GAPDH forward: 5'-CGACCACTTTGTCAAGCTCA-3', GAPDH forward 5'-AGGGGTCTACATGGCAACTG-3'.Relative mRNA expression levels of TINCR were calculated using the $2^{-\Delta\Delta ct}$ methods.

Cell line culture

HCC cells (MHHC97H, MHCC97L and HepG2) and a human hepatocyte (LO2) used in the study were purchased from Shanghai Cell Bank (Shanghai, China). Cells were cultured in Dulbecco's modified Eagle's medium (Gibco, Grand Island, NY, USA) supplemented with 10% fetal bovine Serum (FBS, Invitrogen, CA, USA) and 1% penicillin-streptomycin mixture (Invitrogen, CA, USA) at 37˚C and were maintained in a humidified environment containing 5% CO_2

Cell transfection

Two siRNA-TINCR oligos were constructed and purchased from Ribobio (Guangzhou, China). Cells transfection was performed by using a lipofectamine 2000

reagent (Thermo Fisher Scientific) according to the manufacturer's instructions. The shRNA targeting sequence was as follow: 5'-AATACCTGCTACTTCATGC-3'.

Cell proliferation assay

Cell growth was examined using Cell Counting Kit-8 assay (CCK8, Dojindo Molecular Technologies, Inc., Kunamoto, Japan). Briefly, the 97H and 97L cells at a concentration 2000 cells/well were placed in 96-well plates. CCK-8 reagent was added to each plate to measure cell proliferation at 12, 24, 48 and 72h. The spectrophotometer (Thermo Scientific, Rockford, IL, USA) were used to detect the cell proliferation at the absorbance of 450nm.

Colony formation analysis

Cells were seeded into 12-well plates at a 300 cells/well concentration and then cultured for 14 days in a 5% CO_2 incubator at 37 °C. Furthermore, cells were washed three times by PBS, fixed by 4 % paraformaldehyde and stained with 0.1% crystal violet for 20 min. The number of colony formation in the each filed was counted.

Western blot analysis

Total proteins were extracted with RIPA lysis buffer (Beyotime, China) containing protease inhibitors (Beyotime, China). Equal quantities of proteins were separated by sodium dodecyl sulfate polyacrylamide gel electrophoresi (SDS-PAGE) and then transferred to the polyvinylidene fluoride membraness (PVDF) membrane (Millipore, USA). Next, the membrane was blocked by 5% nonfat milk and incubated with specific antibodies for E-cadherin (Cell Signaling Technology, USA), N-cadherin (Cell Signaling Technology, USA), Ki67 (Cell Signaling Technology, USA), β-actin and (Cell Signaling Technology, USA). The western blot was detected with enhanced chemiluminescence regents (Thermo Scientific, Waltham, MA).

Flow cytometry analysis

Cell cycle was detected using Cell cycle Assay Kit (BD Biosciences, New York, NY, USA). Cells were stained by propidium iodide (PI) in the dark and then incubated at room temperature, the cell cycle were analyzed using a FACS flow cytometer (Accuri C6; BD Biosciences, San Jose, CA, USA).

Statistical analysis

All statistical were conducted by using SPSS 20.0 statistical software package (IBM, Armonk, NY, USA). Results from experiments were shown as mean ± standard deviation (SD). Differences between groups were analyzed using Student's *t*-test. P-value <0.05 was considered statistical significance.

Results

LncRNA TINCR was higher expression in HCC and correlated with tumor size and vascular invasion of HCC patients

We assessed the expression of lncRNA TINCR in 63 HCC tissues specimens and adjacent normal tissues specimens from patients. The qRT-PCR analysis results confirmed that lncRNA TINCR expression was significantly higher in 43 of 62 HCC specimens (**Figure 1A**). According to the median value of lncRNA TINCR expression in HCC specimens, we classified the patients into higher (above the median, n = 34) and lower (below the median, n = 29) lncRNA TINCR expression groups. Furthermore, the association between TINCR expression and clinicopathological characteristics of HCC patients were analyzed. By chi-square test analysis, we observed that Higher TINCR expression was positively correlated with tumor size (**Figure 1B, Table 1**, P=0.015), and vascular invasion (**Figure 1C, Table 1**, P=0.037), while no significant correlation with other factors. In addition, as shown in **Figure 1D**, lncRNA TINCR expression was higher in three HCC cells (97H, 97L and HepG2), compared with LO2 cells. These results indicated that lncRNA TINCR was higher expression in HCC and may be involved in tumor progression.

Downregulation of lncRNA TINCR suppresses cell proliferation and cell cycle progression of HCC in vitro

To explore insight into the functional role of TINCR in HCC cells growth, we knockdown endogenous lncRNA TINCR expression in 97H and 97L cells using two short interfering RNAs (siRNAs) (**Figure 1E**). Compared with control group, lncRNATINCR knockdown significantly inhibited cell viability both in 97H and 97L cell lines after cell transfection at 48 and 72h (**Figure 2A-2B**). Furthermore, cell colonies number was significantly reduced both in 97H and 97L cell lines after lncRNA TINCR knockdown compared to control group, respectively (**Figure 2C-2D**). Moreover, flow cytometry assay confirmed that lncRNA TINCR knockdown increased the G1 phase proportion and reduced S phase proportion both in 97H and 97L cell lines compared with the control group (**Figure 2E-2F**). Collectively, these results indicated that lncRNA TINCR expression profoundly promoted HCC cell proliferation.

Downregulation of lncRNA TINCR suppresses cell invasion and EMT phenomenon of HCC in vitro

Tumor invasion and metastasis play crucial roles in HCC progression and EMT significantly associated with tumor metastasis [13]. To examine the effects of lncRNA TINCR on cell invasion, we performed the tranwell invasion assays. The results showed that lncRNA TINCR silencing obviously impaired cell invasion ability in 97H and 97L cells compared to the control group (**Figure 3A-3B**). Furthermore, we detected the EMT related makers E-cadherin and N-cadherin by western blot analysis by siRNA-TINCR-2 due to its higher silencing efficiency. Western blot analysis

demonstrated that downregulation of lncRNA TINCR markedly increased the E-cadherin expression, while decreased the N-cadherin expression in 97H and 97L cells compared to the control group (**Figure 3C-3D**).

Discussion

Advanced studies had implied that long non-coding RNAs were involved in tumorigenesis and development in different cancers [14]. LncRNAs play distinct roles in the pathogenesis and metastasis of HCC [15]. In the present study, we demonstrated that TINCR expression was significantly up-regulated in HCC tissues and cell lines. Higher TINCR was significantly correlated with tumor size and vascular invasion. In previous study, Tian et al revealed that high lncRNA TINCR expression was significantly correlated with tumor size, tumor differentiation status, TNM stage, and vascular invasion of HCC, which is consistent with our finding in HCC [12]. However, the underlying role of lncRNA TINCR in HCC was not fully elucidated. We demonstrated that lncRNA TINCR knockdown inhibited cell proliferation ability and increased the G1 phase proportion and reduced S phase proportion in HCC cells.

Invasion and metastasis often adumbrate an advanced stage while recurrence often indicates a poor prognosis [16]. Furthermore, we demonstrated that lncRNA TINCR knockdown inhibited cell invasion ability and EMT process by markedly increasing the E-cadherin expression, while decreasing the N-cadherin expression in HCC cells. In gastric cancer, low expression of TINCR increased cell apoptosis and inhibited the proliferation of GC cells [17]. Xu et al showed that E2F1 accelerates GC growth partly through induces TINCR transcription [18]. Zhang et al reported that knockdown of TINCR expression promotes proliferation, metastasis through activating EpCAM cleavage in colorectal cancer [19]. Moreover, to explore whether TINCR knockdown affected cell proliferation in vivo, we performed the tumor formation assay. The results confirmed that knockdown of lncRNA TINCR significantly reduced tumor volume and tumor weigh compared with the empty vector group. Thus, we demonstrated that TINCR knockdown could inhibited cell growth in vivo.

In conclusion, we demonstrated that lncRNA TINCR acted as a promoting tumor role in HCC. Knockdown of lncRNA TINCR inhibited cell proliferation, invasion, and EMT process. In vivo lncRNA TINCR knockdown reduced the tumor growth. Thus, these findings suggested a potential therapeutic value of lncRNA TINCR for HCC.

Disclosure of interest

The authors declare that they have no conflicts of interest.

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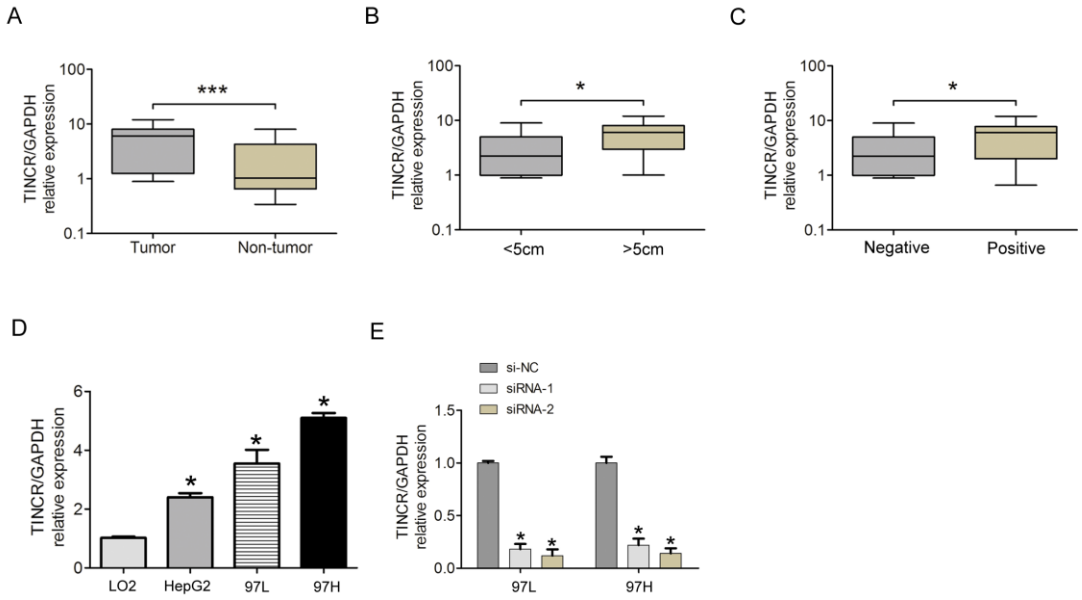
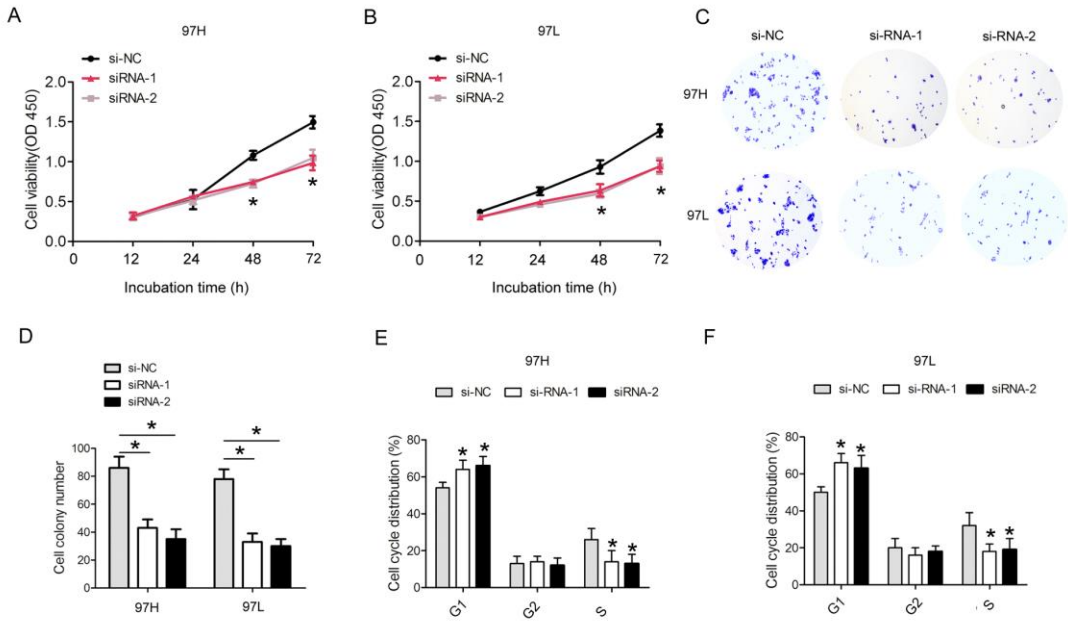
Figure legends

Figure 1



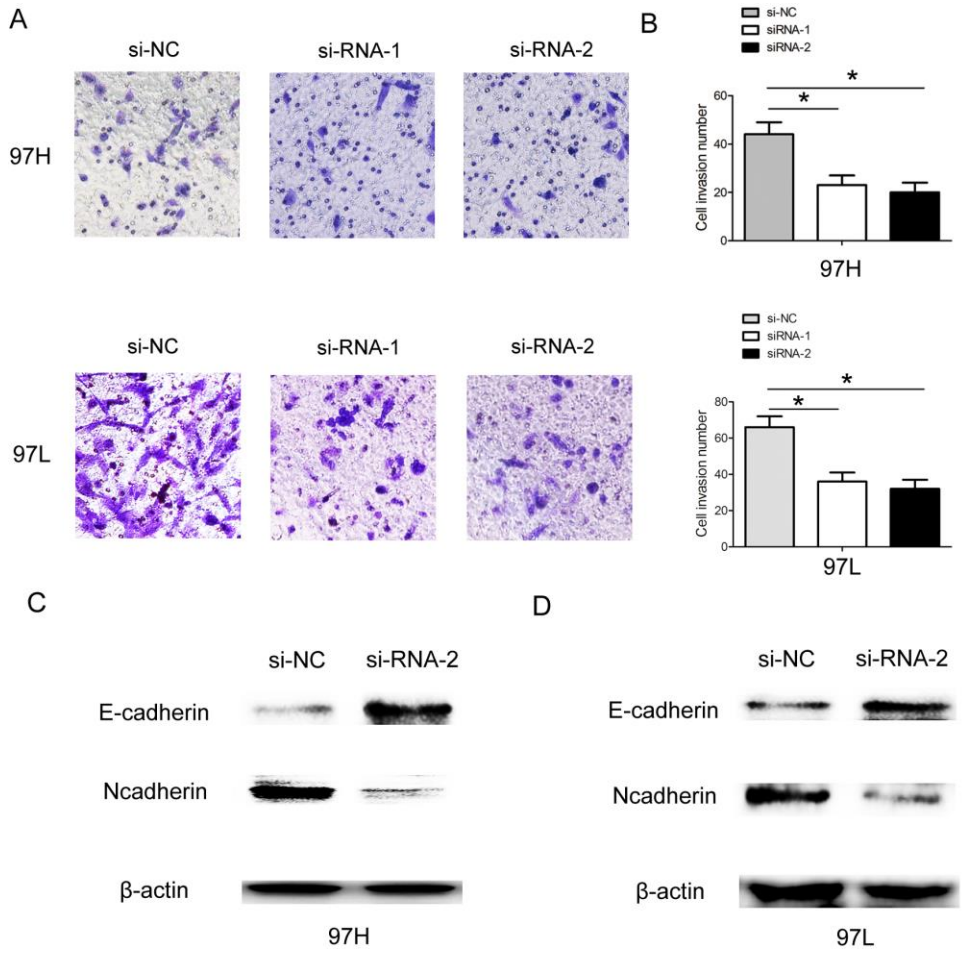
LncRNA TINCR was overexpressed in HCC and associated with tumor size and vascular invasion. (A) LncRNA TINCR expression in HCC tissues and adjacent normal tissues was detected by qRT-PCR. Statistical differences between HCC tissues and paired adjacent normal tissues were analyzed using paired samples t-test (***P<0.001). (B) Association of lncRNA TINCR expression with tumor size of HCC patients was shown. (C) Association of lncRNA TINCR expression with vascular invasion of HCC patients was shown. (D) LncRNA TINCR expression in HCC cells and LO2 cells were detected by qRT-PCR. (E) LncRNA TINCR expression was detected when cells were transfected with si-NC, si-TINCR-1(siRNA-1) or si-TINCR-2 (siRNA-2) in MHCC97H (97H) and MHCC97L (97L) cells. Error bars indicate the mean ± SD. *p < 0.05

Figure 2



LncRNA TINCR knockdown inhibited cell proliferation and cell cycle progression. (A)-(B) CCK8 assay was performed after 97H and 97L cells were transfected with si-NC, si-TINCR-1(siRNA-1) or si-TINCR-2 (siRNA-2) at 12, 24, 48 and 72h. (C)-(D) Cell colony assays were shown and cell colonies number was calculated after 97H and 97L cells were transfected with si-NC, si-TINCR-1(siRNA-1) or si-TINCR-2 (siRNA-2) at 14 days. (E)-(F) cell cycle proportion was shown after 97H and 97L cells were transfected with si-NC, si-TINCR-1(siRNA-1) or si-TINCR-2 (siRNA-2) at 48h. Error bars indicate the mean ± SD. *$p < 0.05$

Figure 3



LncRNA TINCR knockdown inhibited cell invasion and cell EMT process. (A)-(B) Cell invasion assays were shown and cell invasive number was calculated after 97H and 97L cells were transfected with si-NC, si-TINCR-1(siRNA-1) or si-TINCR-2 (siRNA-2) at 48h. (C)-(D) Relative expression of E-cadherin and N-cadherin was shown by western blot after 97H and 97L cells were transfected with si-NC or si-TINCR-2 (siRNA-2) at 48h. Error bars indicate the mean ± SD. *p < 0.05

Table 1 Association of lncRNA TINCR expression with clinicopathologic parameters in 63 HCC patients.

		lncRNA TINCR expression levels		
Clinicopathologic parameters	**N**	**Lower(n=28)**	**Higher(n=35)**	**P-value**
Age(year)				0.624
≤55	45	21	24	
>55	18	7	11	

Gender				0.941
Male	52	23	29	
Female	11	5	6	
Tumor size(cm)				0.015[a]
<5	39	22	17	
>5	24	6	18	
AFP(ng/ml)				0.948
<20	16	7	9	
>20	47	21	26	
Differentiation status				0.806
Well/ Morderately	44	20	24	
Poor	19	8	11	
Liver cirrhosis				0.124
Present	36	19	17	
Absent	27	9	18	
Vascular invasion				0.037[a]
Negative	29	17	12	
Positive	34	11	23	
TNM stage				0.473
I-II	42	20	22	
III-IV	21	8	13	

[a]P<0.05 was considered statistical significance.