Original Article

Long non-coding RNA LINC00673 promotes hepatocellular carcinoma progression and metastasis through negatively regulating miR-205

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Abstract: Increasing evidence demonstrates abnormal expression of long non-coding RNA (lncRNA) is closely correlated with various malignancies including hepatocellular carcinoma (HCC). The present study aims to investigate the role of lncRNA long intergenic noncoding RNA 00673 (LINC00673) in tumorigenesis of HCC. Quantitative real-time polymerase chain reaction (qRT-PCR) revealed LINC00673 was upregulated in HCC cancerous tissue and cell lines compared to adjacent normal tissue and normal liver cell lines. LINC00673 overexpression is associated with poor prognosis and low survival rate. LINC00673 silencing inhibited the proliferation, invasion and epithelial-mesenchymal transition (EMT) of HCC cells in vitro. Bioinformatics analysis revealed that miR-205 targeted 3'-UTR of LINC00673. Rescue experiments confirmed that miR-205 could reverse the effect of LINC00673 on HCC cells. In vivo xenograft tumor assay LINC00673 silencing reduced the tumor volume and weight. Taken together, findings indicate overexpression of LINC00673 promotes HCC cells progression by regulating miR-205, providing a prognostic biomarker and therapeutic target for HCC and is associated with poor survival of HCC patients.

Keywords: Hepatocellular carcinoma, lncRNA, LINC00673, miR-205, EMT, metastasis

Introduction

Hepatocellular carcinoma (HCC) is the fifth most common cancer and the third leading cause of cancer death in the world [1, 2]. The 5-year survival rate for HCC patients is approximately 30% and is characterized by high rates of metastasis resulting in a poor prognosis for patients [3, 4]. The underlying mechanisms of HCC metastasis is not well understood and given the high rates of diagnosis for HCC worldwide and requires further study.

Non-coding RNA (ncRNA) are important regulators involved in most cellular functions and modulation [5]. Typically, ncRNA contains long ncRNA (lncRNA, more than 200 nucleotides), small ncRNA (fewer than 200 nucleotides) and microRNA (miRNA, 18-22 nucleotides). Among these ncRNA, lncRNA participate in a variety of biological and pathological processes, including tumorigenesis [6]. For example, lncRNA CCAT2 expression significantly suppresses glioma cell growth, migration and invasion, and induces early apoptosis of glioma cells [7]. LncRNA also modulates epigenetic transcription regulation and modulates epigenetic post-transcription regulation [8]. The transcription and post-transcription regulation of tumors by lncRNAs is increasing and requires further study.

MicroRNAs (miRNAs) are 18-22 nucleotides in length [9] and participate in tumorigenesis, development, invasion and metastasis [10] and are important regulators in carcinogenesis and HCC [11]. While the exact function of miR-205 is still unclear, it plays a role in de-regulation of a number of cancers [12] and in particular, the genesis and development of HCC.

The present study aims to investigate the role of abnormal LINC00152 expression in HCC progression and metastasis, and further explore the regulation of LINC00152 through negatively regulating miR-205.
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Materials and methods

Tissue collection and ethical statement

This study was approved by Fifth Affiliated Hospital of Guangzhou Medical University. Besides, relevant ethical reviews had been approved Ethics Committee and Institutional Review Board of Guangzhou Medical University. Written consent was obtained from all enrolled patients and healthy controls.

Fifty-three patients with HCC not receiving radiation therapy or chemotherapy at Fifth Affiliated Hospital of Guangzhou Medical University were recruited for the study between May 2014 and Oct 2015. All tissue samples, (including tissues and their paired adjacent normal tissues more than 5 cm away from the tumor border) were collected during surgery, and assessed by histopathological confirmation. After surgery, specimens were immediately frozen at -80°C for further use.

HCC cells and culture

Human HCC cell lines (HCCLM3, MHCC97L, HepG2, Hep3B and Huh7) and normal liver cell lines (THLE-3, L-02) were provided by the Chinese Academy of Sciences Cell Bank (Shanghai, China). All cells were cultured in Dulbecco’s Modified Eagle’s Medium (DMEM, Gibco, USA) containing 10% fetal bovine serum (FBS, HyClone, USA) and 1% penicillin/streptomycin. The culture condition was kept at 5% CO2 at 37°C.

Real-time qPCR

Total RNA was isolated from cells using TRIzol reagent (InVitrogen) according to the manufacturer’s instructions. The isolated RNA (10 μg) was used to synthesize cDNA using RevertAid™ firs Strand cDNA Synthesis Kit (Fermentas, Germany). PCR was performed using quantitative SYBR Green PCR kit (TaKaRa, Dalian, China) according to the manufacturer’s protocol. GAPDH was used as internal control. All primers were designed and synthesized by GenePharma (Shanghai, China) and shown as follows: LINC00673, forward, 5’-AAATTAAAGGGTCCAGTCCTACA-3’; reverse, 5’-TTCCGATCAGTTGGAAT-3’; Relative levels of gene expression were expressed relative to GAPDH and calculated using the 2ΔΔCT method.

RNA interference transfection

All siRNAs and negative controls were designed and purchased from GenePharm company (Shanghai, China). The siRNA sequences were as follows: si-LINC00673-1, 5’-CGGGAUGAACAUAGACAGCA-3’; si-LINC00673-2, 5’-CCAUCUAUCUAUCUCUA-3’; si-miR-205, 5’-CUCAUCAUCAUCAUCAUCAUCA-3’. Interfering RNA plasmids (20 nM) were transfected into cells in 6-well plates using Lipofectamine™ 2000 reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer’s instructions.

Cell proliferation assay

The proliferation of HCC cells was measured using MTT assay. HCC cells (MHCC97L and HepG2) at logarithmical growing phase were seeded into 96-well culture plates at density of 1×10^4 cells per well with DMEM medium (Hyclone). MTT (0.5 mg/ml) was added into each well and incubated at 37°C. DMEM was then changed to 100 μl DMSO for incubation at room temperature. The absorbance was detected at 490 nm.

Colony formation assay

For the colony formation assay, HCC cells were seeded into 6-well plates at a density of 5×10^3 cells per well. Cells were then cultured at 37°C with 5% CO2 and cultured for 2 weeks. Finally, colonies were fixed with 4% paraformaldehyde and stained with 0.1% crystal violet (Beyotime, Shanghai, China) for 30 min. The number of colonies was counted on light microscopy and the experiment was performed in triplicate.

Transwell invasion assay

Invasion assay of HCC cells was performed with Transwell invasion assay according to the manufacturer’s instructions (BD Biosciences, San Jose, CA, USA). Briefly, MHCC97L and Hepg2 cells at density of 1×10^4 per well were respectively seeded into the upper chamber filled with medium containing 1% FBS. The lower chamber was filled with culture medium with 10% FBS medium. After 24 hours incubation, cells
adhered on the upper surface of membrane were removed, while the cells migrated into the lower chamber were stained with 0.1% crystal violet. All experiments were performed in triplicate.

Western blot analysis

Total proteins were lysed by RIPA buffer (Sigma-Aldrich), containing protease inhibitors cocktail (Roche, USA). After centrifugation (12,000 g for 5 minutes), protein concentrations were detected with Bio-Rad Protein Assay Kit (Bio-Rad Laboratories, CA, USA). SDS-PAGE was conducted to separate equal amounts of cellular proteins, which were blotted onto PVDF membrane (Millipore, Billerica, MA, USA) and incubated overnight at 4°C with primary antibodies. Primary antibodies were purchased from Abcam Company shown as follows: anti-E-cadherin (1:1000), anti-vimentin (1:1000), and anti-N-cadherin (1:1000). The membrane was incubated with second antibody (horseradish peroxidase-conjugated goat anti-rabbit) at 4°C overnight. Finally, the blots were detected using an EZ-ECL chemiluminescence Detection kit for HRP (Biological Industries, Beit-Haemek, Israel).

Luciferase reporter assay

The 3'-UTR of LINC00673 containing miR-205 binding sites was amplified and cloned into the pGL3-basic luciferase vector (Promega, USA). HEK-293T cells were then seeded in 96-well plates for 24 h culture before transfection with the LINC00673 wild-type or mutant reporter vector, miR-205 mimics or negative control and Renilla plasmid using Lipofectamine 2000 (Invitrogen, Carlsbad, Calif, USA). Luciferase activity was detected after transfection 24 h using Dual-Luciferase Reporter System (Promega, Madison, WI, USA). Renilla luciferase was used as an internal reference. All experiments were performed in triplicate and repeated three times.

In vivo xenograft tumor growth

A total of 10 male BALB/C nude mice (5 week, 5 mice in control group and 5 mice in si-LINC00673 group) were used for the xenograft tumor growth assay. MHCC97L cells (5×10⁵) were injected subcutaneously into the right flank of mice. After injection, the tumor volume was tested every five days according to the formula: Volume = 0.5×length × width × width. After 25 days, mice were sacrificed and the tumor was weighed. The animal experiment was approved by the Committee on the Use of Live Animals in Teaching and Research of Fifth Affiliated Hospital of Guangzhou Medical University.

Statistical analysis

Statistical analysis was performed using SPSS (vision, 19.0; SPSS, Inc., Chicago, IL, USA) and
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GraphPad (vision 6.0, USA). Values are represented as mean ± standard deviation (SD). Student’s t-test or one-way ANOVA were used for comparisons between groups. Kaplan-Meier’s method and the log-rank test were performed for survival rate. P<0.05 was identified as significant difference.

Results

**LINC00673 was up-regulated in HCC tissue and correlated with poor prognosis**

In the early phase of experiments, 53 HCC tissue and adjacent normal tissues samples were collected and RT-PCR was performed to measure the expression level of LINC00673. Results showed that LINC00673 expression levels were significantly up-regulated in HCC tissue compared to adjacent normal tissues (Figure 1A). The HCC tissues samples were divided into two groups according to the LINC00673 median expression; they were a high LINC00673 expression group and a low LINC00673 expression group (Figure 1B). Moreover, LINC00673 expression was significantly higher in patients with advanced clinical stage disease (III-IV phase) than those with early clinical stage disease (I-II phase) (Figure 1C). Kaplan-Meier curves of overall survivals and log-rank test showed that HCC patients
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with high LINC00673 levels had lower overall survival rates than those with low LINC00673 level (P=0.0128) (Figure 1D). Taken together, LINC00673 was up-regulated in HCC tissue and correlated with poor prognosis, suggesting the oncogenic role of LINC00673 in HCC genesis.

LINC00673 silencing suppressed the proliferation and invasion of HCC cells in vitro

Subsequent analysis, found LINC00673 expression level was significantly up-regulated in five HCC cell lines compared to two normal cell lines (Figure 2A). To determine the biological function of LINC00673, LINC00673 silencing via siRNA transfection in HCC cells in MHCC97L and HepG2 cell lines in vitro was established (Figure 2B). Colony formation assay found LINC00673 silencing markedly decreased the clone number in MHCC97L and HepG2 cell lines compared to the negative control group (Figure 2C). MTT assay indicated LINC00673 silencing inhibited the proliferation of MHCC97L and HepG2 cells (Figure 2D, 2E). Transwell assay revealed LINC00673 silencing inhibited the invasive ability of HCC cells (Figure 2F, 2G). Overall, results demonstrated the inhibitory function of LINC00673 silencing on HCC cells in vitro, suggesting the underlying role of LINC00673 in HCC tumorigenesis.

LINC00673 silencing regulated epithelial-mesenchymal transition (EMT) related markers expression

E-cadherin, N-cadherin and vimentin are EMT maker proteins that reflect the metastasis of tumor tissue. To verify the role of LINC00673 silencing...
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on HCC metastasis, the expression of E-cadherin, N-cadherin and vimentin was measured using Western blot analysis. Results found LINC00673 silencing significantly increased E-cadherin expression in MHCC97L and HepG2 cells, while N-cadherin and vimentin expression was decreased (Figure 3A-D). Results showed that LINC00673 silencing decreased the metastasis of HCC cells.

LINC00673 was targeted by miR-205 at 3'-UTR

The potential targeting miRNAs binding with LINC00673 were screened using bioinformatics analysis and miR-205 was found to closely target 3'-UTR of LINC00673. The 3'-UTR binding sites were shown as follows (Figure 4A). Luciferase reporter assay confirmed the binding with the decreasing fluorescence within miR-205 mimics and LINC00673 wild type (Figure 4B). Expression of miR-205 in HCC tissue was decreased compared to adjacent normal tissue (Figure 4C). Moreover, after HCC cells (MHCC97L and HepG2) were transfected with si-LINC00673, miR-205 expression levels were significantly up-regulated (Figure 4D). Overall, results suggest miR-205 targeted LINC00673 at 3'-UTR and was negatively correlated with LINC00673 expression.

MiR-205 reversed the function of LINC00673 in MHCC97L cells

It had been found that miR-205 targeted LINC00673 3'-UTR and was negatively correlated with LINC00673 expression. Expression of miR-205 was significantly down-regulated in HCC cells (Figure 5A). In these cell lines, MHCC97L cells were used to perform rescue experiments to verify the role of miR-205 compared to LINC00673. Results found miR-205 inhibitor decreased the expression of miR-205 induced by si-LINC00673 (Figure 5B). Colony formation assay and MTT assay revealed miR-205 inhibitor suppressed the proliferation of MHCC97L cells induced by si-LINC00673 (Figure 5C, 5D). Similarly, transwell assay showed miR-205 inhibitor suppressed the invasion of MHCC97L cells induced by si-LINC00673 (Fi-
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General results showed miR-205 could reverse the function of LINC00673 on HCC cells, suggesting the antergic interaction within miR-205 and LINC00673.

LINC00673 silencing could inhibit tumor growth in vivo

To investigate the role of LINC00673 on HCC tumor growth, we performed a xenograft model experiment in vivo. Results found LINC00673 silencing (si-LINC00673) markedly decreased the tumor volumes and weights compared to the control group (si-NC) (Figure 6A, 6B). Results revealed LINC00673 silencing suppressed tumor growth in vivo, suggesting the tumor promoting role of LINC00673 on HCC.

Discussion

Hepatocellular carcinoma (HCC) is a serious malignant tumor characterized by metastasis and postsurgical recurrence, and thereby poor prognosis and low survival rates [13, 14]. Long non-coding RNAs (lncRNAs) have been found to participate in the tumorigenesis of HCC [15]. The present study found that LINC00673 was up-regulated in HCC tissue and cells, and modulated the HCC carcinogenesis via harboring miR-205.

The proliferation, invasion and metastasis of tumor cells are the key characteristics affecting the progression and recurrence of tumor tissue [16]. The present study found greater expression of LINC00673 predicted a poorer prognosis for HCC patients. Loss-of-function experiments reveal that LINC00673 silencing inhibited HCC cell (MHCC97L and HepG2) proliferation and invasion in vitro. Furthermore, LINC00673 silencing increased E-cadherin expression, and decreased vimentin and N-cadherin expression, suggesting the EMT inhibiting role of LINC00673 knockdown on HCC cells. The results suggest pathological overexpression of LINC00673 increases the progression and metastasis of HCC tissue, and LINC00673 knockdown suppresses tumorous development.

LncRNA has been identified as an important regulator in the epigenetic mechanism involved in histone modification, DNA methylation, transcriptional regulation and post-transcriptional regulation [17, 18]. Given the extensive distribution and multidimensional function of IncRNAs additional research is required on this topic [19]. LncRNA acts as regulator in the tumorigenesis and progression of HCC [20]. For example, IncRNA MALAT1 is up-regulated in HCC tissue and is found to regulate expression of hepatitis B virus X protein [21]. LncRNAs also modulate DNA methylation and affect gene expression and phenotypes. For example, IncRNA PCAT-14 is overexpressed in HCC tissue and is found to regulate expression of ATAD2 and activation of the Hedgehog pathway by inducing methylation of the miR-372 promoter [22].

The regulation mechanism of IncRNA functions to adsorb target miRNAs to indirectly modulate the functional gene expression, this is also known as competing endogenous RNA (ceRNA) or miRNA ‘sponge’ [23]. Using a bioinformatics prediction program, the present study described the potential downstream miR-205 targeting with LINC00673 3'-UTR. Rescue experiments suggested the effective binding within...
miR-205 and LINC00673, validating the regulating approach of LINC00673 harboring miR-205. Given these results, it is suggested that LINC00673 plays a regulatory role in HCC tumorigenesis. It has been reported that miR-205 is significantly down-regulated in HCC tissue, which modulates HCC genesis via targeting PLCβ1 gene [24].

The mechanism of ceRNA is involved in tumorous genesis, development, progression and metastasis [25]. In glioma cancer tissue, IncRNA PVT1 negatively regulates miR-424 expression to act as an oncogenic IncRNA in tumorigenesis [26]. In HCC, IncRNA HULC depletion inhibited the growth and metastasis of HCC cell lines in vitro and in vivo, suggesting HULC regulates ZEB1-induced EMT via HULC/miR-200a-3p/ZEB1 signaling pathway [27].

The present study demonstrates the role of IncRNA LINC00673 in HCC progression and metastasis via harboring miR-205, providing a novel insight for pathogenesis and highlighting a therapeutic target for HCC.

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Disclosure of conflict of interest

None.

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