

LINC00265 Promotes Metastasis and Progression of Hepatocellular Carcinoma by Interacting with E2F1 at The Promoter of *CDK2*

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Abstract

Objective: This study aimed to explore biological function of long intergenic non-protein coding RNA 265 (LINC00265) in hepatocellular carcinoma (HCC) cells, and evaluate its potential function as a biomarker.

Materials and Methods: In this experimental study, GEPIA database and Kaplan-Meier Plotter database were employed to analyze LINC00265 expression in HCC tissue samples and its predicting value for prognosis. LINC00265 expression in HCC tissues and cells was detected by quantitative real-time polymerase chain reaction (qRT-PCR). After overexpressing and knocking-down of LINC00265 in HCC cells, cell counting kit-8 (CCK-8) and 5-Ethynyl-2'-deoxyuridine (EdU) assays were adopted to detect proliferation of HCC cells. Transwell assay was used to detect migration and invasion of HCC cells. Interaction of LINC00265 with E2F transcription factor 1 (E2F1) was verified by the catRAPID online analysis tool, RNA pull-down experiment and RNA binding protein immunoprecipitation (RIP) assay. Binding of E2F1 to the promoter region of cyclin-dependent kinases 2 (*CDK2*) was detected by dual-luciferase reporter assay and chromatin immunoprecipitation. Regulatory effects of LINC00265 and E2F1 on *CDK2* expression were probed by Western blot.

Results: LINC00265 expression was increased in HCC tissues and cells. LINC00265 overexpression promoted proliferation, migration and invasion of HCC cells, and knocking-down LINC00265 worked oppositely. LINC00265 could bind to E2F1 and it could enhance combination of E2F1 and *CDK2* promoter regions, thus promoting *CDK2* transcription. LINC00265 overexpression promoted expression of *CDK2* in HCC cells.

Conclusion: Our data suggested that LINC00265 can promote malignant behaviors of HCC cells by recruiting E2F1 to the promoter region of *CDK2*.

Keywords: Cyclin-Dependent Kinases 2, E2F Transcription Factor 1, Hepatocellular Carcinoma, LINC00265, Proliferation

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Introduction

Liver cancer is identified as the sixth most prevailing tumor in the globe and the fourth-largest cause of cancer-related death, among which hepatocellular carcinoma (HCC) accounts for about 90% cases (1). At present, the main treatment methods for HCC include surgical resection, liver transplantation and radiofrequency ablation (2). Although treatment for HCC has progressed in recent years, the overall 5-years survival rate of HCC patients is only about 7% (3). Therefore, it is particularly important to explore pathogenesis of HCC as well as providing new ideas and targets for clinical diagnosis and treatment of HCC.

Long non-coding RNAs (lncRNAs), recognized as eukaryotic transcripts with over 200 nucleotides in length, are devoid of protein-coding ability. lncRNA regulates gene expression at the transcriptional and post-transcriptional levels, thus participating in regulating some biological processes related to diseases (4, 5). The scientific consensus is that a variety

of lncRNAs are abnormally expressed in various tumors and lncRNAs can serve as tumor promoters or suppressors to participate in the progression of tumors (6-8). The cancer genome atlas (TCGA) database shows that long intergenic non-protein coding RNA 265 (LINC00265), an important member of lncRNA family, is overexpressed in colorectal cancer (CRC) tissues, which is associated with the poor prognosis of CRC patients; knocking-down of LINC00265 inhibited viability and invasion of CRC cells, induced cell cycle arrest and promoted apoptosis (9). Another study reported that LINC00265 overexpression promoted glycolysis and lactic acid production of CRC cells by up-regulating TRIM44 expression, thus accelerating growth of the tumor cells (10). Instead, biological function and molecular mechanism of LINC00265 in HCC warrant further investigation.

E2F transcription factor 1 (E2F1) is involved in regulating various biological processes, including cell proliferation, differentiation and apoptosis. E2F1 can potentiate the malignant biological behaviors of HCC

cells (11). Cyclin-dependent kinases 2 (*CDK2*) is vital in cell cycle regulation and partakes in a series of biological processes, including DNA damage repair, intracellular transport, protein degradation, signal transduction, DNA and RNA anabolism and translation (12). Previous studies showed that knocking-down of *CDK2* can effectively inhibit the progression of lung cancer, breast cancer and HCC (13-15).

The present study aimed to investigate the role and mechanism of LINC00265/E2F1/CDK2 axis in HCC.

Materials and Methods

Tissue samples

In this experimental study, with the approval of the Ethics Committee of Guangxi Zhuang Autonomous Region Brain Hospital (Guangxi, China; Approval No. 20160105) and the informed consent of all patients, we selected 46 HCC patients who were treated in Guangxi Zhuang Autonomous Region Brain Hospital and collected the resected cancer tissues and their corresponding adjacent tissues, respectively. The tissues were stored at -80°C after surgical resection. All patients had complete clinical and clinicopathological data. They did not receive any neoadjuvant treatment, such as radiotherapy or chemotherapy, before the surgery.

Cell culture and transfection

Human normal hepatocytes (THLE-3) and HCC cell lines (BEL-7402, Hep3B, and Huh7) were purchased from the Cell Center of the Chinese Academy of Sciences (Shanghai, China). HCC cell line HCCLM3 was obtained from the China Center for Type Culture Collection, (CCTCC, Wuhan, China). The cells were inoculated into Roswell Park Memorial Institute (RPMI)-1640 medium (Thermo-Fisher Scientific, USA) with 10% fetal bovine serum (FBS, Thermo-Fisher Scientific, USA), and cultured at 37°C in 5% CO_2 . The cells were sub-cultured every 2-3 days and the cells in logarithmic growth phase were harvested for the subsequent experiment. E2F1 overexpression plasmid (oe-E2F1), *CDK2* overexpression plasmid (oe-*CDK2*), LINC00265 overexpression plasmid (oe-LINC00265), negative control of overexpression plasmids (oe-NC), siRNAs targeting *CDK2* (si-*CDK2*), siRNAs targeting E2F1 (si-E2F1) and negative control of siRNA (si-NC) were constructed by GenePharma (Shanghai, China). For transfection, BEL-7402 and Hep3B cells were transferred into a 6-well cell plate at a density of 3×10^5 cells/well. They were cultured at 37°C in 5% CO_2 for 24 hours. Subsequently, the cells were transfected by Lipofectamine[®] 3000 (Invitrogen, USA). Transfection efficiency was verified by quantitative real-time polymerase chain reaction (qRT-PCR) and Western blot after 48 hours.

Quantitative real-time polymerase chain reaction

Total RNA of tissues and cells was extracted by TRIzol reagent (Invitrogen), followed by detection of purity and reverse transcription into cDNA. Then, qRT-PCR was

performed with SYBR[®]Premix-Ex-Taq[™] (Takara, Japan) on the ABI7300 system (Thermo-Fisher Scientific, USA). Relative expression of LINC00265 was calculated by $2^{-\Delta\Delta\text{Ct}}$ method using GAPDH as the internal reference. The utilized primer sequences are as following:

LINC00265-

F: 5'-GGAAGAGAGACTGACTGGGC-3'

R: 5'-GTTTCGCTGTCACCCCTCTG-3'

GADPH-

F: 5'-GTCAACGGATTTGGTCTGTATT-3'

R: 5'-AGTCTTCTGGGTGGCAGTGAT-3'

Cell counting kit-8 (CCK-8) assay

BEL-7402 and Hep3B cells, in the logarithmic growth phase, were trypsinized by trypsin, with the cell density adjusted to 2×10^4 cells/ml. The cells were then inoculated into 96-well plates with 100 μl of cell suspension per well, followed by culturing for 24, 48, 72 and 96 hours, respectively. After that, the cells were incubated with 10 μl CCK-8 solution (Dojindo Molecular Technologies, Japan). After 1 hour, value of optical density at 450 nm ($\text{OD}_{450\text{nm}}$) was measured by a microplate reader.

5-Ethynyl-2'-deoxyuridine (EdU) experiment

BEL-7402 and Hep3B cells in the experimental and the control groups were transferred into the 24-well plate, incubated with 200 μl of 5 $\mu\text{mol/L}$ EdU medium (Beyotime Biotechnology, China) for 2 hours and then immersed in phosphate buffered saline (PBS). Next, the cells were fixed with paraformaldehyde for 10 minutes at ambient temperature and incubated with 200 μl of 2 mg/ml glycine for 5 minutes. They were subsequently immersed in PBS on the shaking table for 5 minutes. After that, 100 μl PBS with 0.5% Triton X-100 was dripped into each well, followed by incubation of them for 10 minutes on a shaking table and rinsing with PBS twice for 5 minutes each time. Subsequently, the above cells were stained with Apollo at room temperature for 30 minutes and then incubated with $1 \times$ Hoechst 33342 DNA staining solution for 20 minutes. After washing with PBS, the cells were observed and number of the cells was counted under a fluorescent microscope.

Transwell experiment

BEL-7402 and Hep3B cells, trypsinized with 0.25% trypsin, were centrifuged, resuspended and then dispersed. Matrigel (Corning, China) was adopted for invasion experiments, but not for migration experiments. The transfected cells (5×10^4 cells/well) were transferred into the upper compartment of the Transwell chamber and then 500 μl of complete medium containing 10% serum was added to the lower compartment, with which the cells were cultured at 37°C for 24 hours. Then, the cells which failed to migrate or invade were removed from the upper surface of the membrane. The cells which passed through membrane were fixed with 4% paraformaldehyde for 10

minute, and stained with 0.5% crystal violet. After rinsing with tap water, the membrane was observed under an inverted microscope and number of the cells was counted.

Subcellular fractionation

The NE-PER™ Nuclear and Cytoplasmic Extraction Reagent (Thermo-Fisher Scientific, USA) was used to isolate and collect cytoplasmic and nuclear fractions of Hep3B and BEL-7402 cells. Then, RNA expression levels of LINC00265, U6 (nuclear control transcript) and GAPDH (cytoplasmic control transcript) were analyzed by qRT-PCR.

RNA pull-down assay

To detect interaction of LINC00265 with E2F1, RNA pull-down assay was performed using a Magnetic RNA-Protein Pull-Down Kit (Thermo Fisher Scientific, USA). The protein extracts of HCC cells mixed with biotinylated LINC00265 were incubated with magnetic beads. Ultimately, the eluted proteins from magnetic beads were harvested, and Western blot was performed to detect the protein enrichment.

RNA binding protein immunoprecipitation (RIP) experiment

RIP assay was performed with a Magna RIP™ RIP kit (Millipore, USA). Briefly, BEL-7402 and Hep3B cells in the logarithmic growth phase were suspended with RIP buffer to prepare the cell lysate. Then the lysate was incubated with magnetic beads coupled with anti-E2F1 antibody and negative control IgG. Next, RNA in the lysate was immunoprecipitated and purified and cDNA was obtained by reverse transcription. LINC00265 immunoprecipitation enrichment was detected by qRT-PCR.

Chromatin immunoprecipitation (ChIP) assay

Briefly, BEL-7402 and Hep3B cells were fixed with formaldehyde for 10 minutes. The ultrasonic breaker was set to 10 seconds per ultrasonic cycle with 10-second intervals with 15 cycles to break the chromatin. The product was centrifuged at 12000 g at 4°C for 10 minutes. The supernatant was collected and divided into two test tubes, which were incubated with mouse IgG (1:100, Abcam, China) or anti-E2F1 antibody (2 µg/ml of cell lysate, Abcam, China) at 4°C for overnight. Then, DNA-protein complex was precipitated by Protein Agarose/Sepharose. Subsequently, the cross-linking was reversed overnight at 65°C and DNA fragments were extracted by phenol/chloroform method. Next, qRT-PCR was conducted to amplify *CDK2* sequence with the *CDK2*-specific primers and determine E2F1 binding to the *CDK2* promoter region.

Dual-luciferase reporter assay

Briefly, wild-type *CDK2* and mutant *CDK2* target fragments were constructed and integrated into the pGL3 vector (Promega, USA) to construct pGL3-*CDK2*-wild type (*CDK2*-WT) and pGL3-*CDK2*-mutant (*CDK2*-MUT) reporter vector. BEL-7402 and Hep3B cells were

co-transfected with *CDK2*-WT (or *CDK2*-MUT) and oe-NC (or oe-E2F1). After 48 hours, luciferase activity was determined. The firefly luciferase activity was normalized to Renilla luciferase activity.

Western blot

Total protein of the cells was extracted with RIPA lysis buffer (Beyotime, China), with the protein concentration determined by the BCA method. After denaturation, the protein samples were loaded into each well (20 µg per lane), separated by SDS-PAGE and electrically transferred to the polyvinylidene fluoride (PVDF) membrane. After that, the membrane was blocked with tris buffered saline with tween 20 (TBST) solution containing 5% skimmed milk at ambient temperature for 1 hour. The PVDF membrane was then incubated with the primary antibodies including anti-*CDK2* antibody (1:1000), anti-E2F1 antibody (1:1000) and anti-GAPDH antibody (1:1000, all from Abcam, China) overnight at 4°C, followed by washing in TBST for 4 times, each time for 8 minutes. Next, the PVDF membrane was incubated with the corresponding secondary goat anti-rabbit antibody (1:2000, Abcam, China) for 1.5 hours at room temperature, followed by rinsing with TBST 4 times, each time for 8 minutes. Ultimately, X-ray imaging was performed with ECL Western blot Substrate kit (Thermo-Fisher Scientific, USA) to show the protein bands.

Statistical analysis

All experiments were conducted in triplicate. All data were analyzed by SPSS 20.0 statistical analysis software (SPSS Inc., Chicago, IL, USA). Data were expressed by mean ± standard deviation ($x \pm SD$). To compare two groups, one-sample Kolmogorov-Smirnov test was used to test for normality distribution. For the data which were normally distributed, an independent sample t test was used. For the data with skewed distribution, Mann-Whitney test was used. To make the comparison among three or more groups, a One-way ANOVA test was used, and if there was a significant difference, Turkey's post-hoc test was performed to make the comparison between the two groups. For enumeration data, they were expressed in a contingency table, and subsequently chi-square test and Fisher's exact test were adopted for the comparison of the two groups. Statistically, $P < 0.05$ was considered significant.

Results

LINC00265 was highly expressed in HCC

By searching GEPIA database (<http://gepia.cancer-pku.cn/>), we observed that LINC00265 expression was observably raised in the HCC tissue samples, compared to the normal liver tissues (Fig.1A). Consistently, qRT-PCR showed that LINC00265 expression in the HCC tissues was significantly elevated against the adjacent tissues (Fig.1B). Receiver operating characteristic (ROC) curve analysis was used to evaluate diagnostic accuracy of LINC00265. The results suggested that LINC00265 could be considered as a discriminative biomarker for HCC with 97.83% sensitivity and 91.30% specificity in the optimal cutoff value of 1.49

(AUC=0.98, $P < 0.001$, Fig. 1C). Additionally, GEPIA database and Kaplan-Meier Plotter database (<http://kmplot.com/>) showed that high expression of LINC00265 was associated with shorter disease-free survival time of HCC patients (Fig. S1A, B, See Supplementary Online Information at www.celljournal.org). Furthermore, LINC00265 expression in

four HCC cell lines, compared to that in THLE-3 cells, was markedly up-regulated (Fig. 1D). Chi-square test highlighted that LINC00265 expression was associated with increased tumor node metastasis (TNM) stage of the patients (Table 1), which indicated that LINC00265 might be pivotal in facilitating cancer progression in HCC.

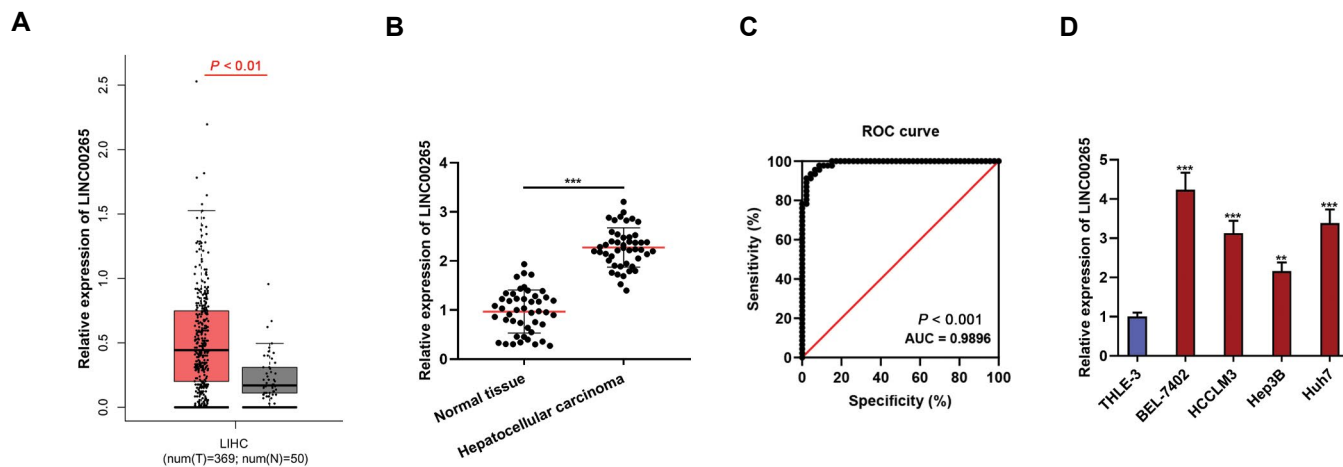


Fig. 1: Expression characteristics of LINC00265 in HCC tissues and cells. **A.** Expression of LINC00265 in HCC tissues (red column, $n=369$) and normal tissues (grey column, $n=50$) was analyzed by the GEPIA database. **B.** Expression of LINC00265 in HCC tissues ($n=46$) and normal tissues ($n=46$) was detected by qRT-PCR. **C.** ROC analysis of the value of LINC00265 expression for distinguishing HCC tissues from normal tissues. **D.** Expression of LINC00265 in human normal liver cells THLE-3 and four human HCC cell lines (BEL-7402, HCCLM3, Hep3B and Huh7) was detected by qRT-PCR. All experiments were repeated three times with three replicates for each repeat. LHC; Liver hepatocellular carcinoma, T; Tumor, N; Normal, HCC; Hepatocellular carcinoma, qRT-PCR; Quantitative real-time polymerase chain reaction, ROC; Receiver operating characteristic, **, $P < 0.01$, and ***, $P < 0.001$.

Table 1: Association of clinicopathological features with expression of LINC00265 in HCC tissues

Pathological parameters	Number ($n=46$)	LINC00265 expression		χ^2	P value
		High ($n=23$)	Low ($n=23$)		
Gender				0.5111	0.4746
Male	36	19	17		
Female	10	4	6		
Age (Y)				2.6807	0.1015
<50	13	4	9		
≥ 50	33	19	14		
Cirrhosis				1.0952	0.2953
Absent	42	22	20		
Present	4	1	3		
Tumor size (cm)				2.4731	0.1158
<5	15	5	10		
>5	31	18	13		
TNM stage				7.2632	0.0070*
I+II	19	5	14		
III+IV	27	18	9		
Tumor multiplicity				0.3538	0.5519
Single	20	9	11		
Multiple	26	14	12		
Degree of differentiation				1.4603	0.2269
Low, medium	28	16	12		
High	18	7	11		

HCC; Hepatocellular carcinoma, TNM; Tumor node metastasis, and *, $P < 0.05$.

Effects of LINC00265 on the malignant biological behaviors of HCC cells

To pinpoint biological function of LINC00265 in HCC cells, Hep3B and BEL-7402 cells were used for subsequent experiments. We transfected LINC00265 overexpression plasmid into Hep3B cells. We also transfected si-LINC00265#1 and si-LINC00265#2 into BEL-7402 cells. Transfection efficiency verified by qRT-PCR (Fig.2A). CCK-8, EdU and Transwell assays indicated that as against the control group, LINC00265 overexpression demonstrably promoted proliferation, migration and invasion of Hep3B cells, while knocking-down LINC00265 worked oppositely in BEL-7402 cells (Fig.2B-F).

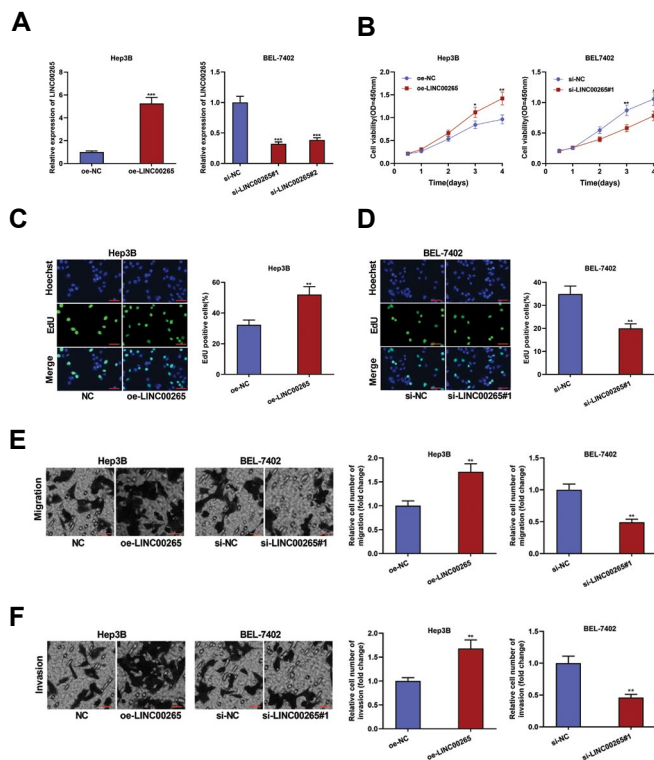


Fig.2: Effects of LINC00265 on proliferation, migration and invasion of HCC cells. **A.** LINC00265 overexpression plasmids and si-LINC00265 were transfected into Hep3B and BEL-7402 cells, respectively. The transfection efficiency was examined by qRT-PCR. **B-D.** After transfection, CCK-8 and EdU assays were used to detect proliferation of Hep3B and BEL-7402 cells. **E, F.** After transfection, Transwell assay was used to detect migration and invasion of Hep3B and BEL-7402 cells (scale bars: 50 μ m). All experiments were repeated three times with three replicates for each repeat. HCC; Hepatocellular carcinoma, qRT-PCR; Quantitative real-time polymerase chain reaction, *, $P < 0.05$, **, $P < 0.01$, and ***, $P < 0.001$.

Interaction of LINC00265 with E2F1

Data from the LncMAP database (<http://bio-bigdata.hrbmu.edu.cn/LncMAP/>) predicted that LINC00265 could promote *CDK2* transcription by recruiting E2F1 to the promoter region of *CDK2* gene (Fig.3A). qRT-PCR showed that LINC00265 mainly existed in the nucleus of HCC cells (Fig.3B), suggesting it may function as a transcriptional

regulator. Then, we searched catRAPID database to predict binding domain of LINC00265 and E2F1. It was determined that LINC00265 might interact with E2F1 (Fig.3C, D). RNA pull-down assay indicated that E2F1 was highly expressed in the LINC00265 biotinylated protein group, but not in the NC-biotinylated group (Fig.3E). RIP assay showed that LINC00265 was remarkably enriched by anti-E2F1 antibody both in Hep3B and BEL-7402 cells, compared to the IgG group (Fig.3F). Collectively, these data suggested that LINC00265 could interact with E2F1 to regulate its biological function.

A

Cancer type	LncRNA ID	LncRNA Symbol	TF ID	TF Symbol	Gene ID	Gene symbol	Mediated pattern	Detail
LHC	ENSG00000188185	LINC00265	ENSG00000191412	E2F1	ENSG00000123374	CDK2	***	more

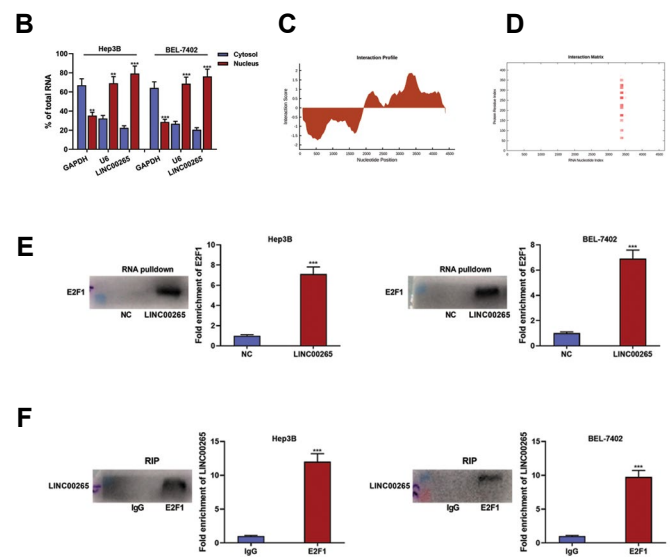


Fig.3: Interactions of LINC00265 with E2F1. **A.** LncMAP database was adopted to predict the binding relationship among LINC00265, E2F1 and *CDK2*. **B.** Subcellular localization of LINC00265 in BEL-7402 and Hep3B cells was detected by qRT-PCR. **C.** Interaction profiles between LINC00265 and E2F1 were predicted by catRAPID database. **D.** Interaction matrix between LINC00265 and E2F1 was predicted by catRAPID database. **E.** Interaction between LINC00265 and E2F1 in BEL-7402 and Hep3B cells was detected by RNA pull-down assay. **F.** Interaction between LINC00265 and E2F1 in BEL-7402 and Hep3B cells was validated by RIP assay. All experiments were repeated three times with three replicates for each repeat. qRT-PCR; Quantitative real-time polymerase chain reaction, **, $P < 0.01$, and ***, $P < 0.001$.

LINC00265 promoted *CDK2* transcription by recruiting E2F1

We then analyzed Promo Database and found that E2F1 could bind to the *CDK2* promoter sequence (Fig.4A). The data in StarBase database (<http://starbase.sysu.edu.cn/>) showed that, in HCC samples, E2F1 expression was positively interrelated with *CDK2* expression and LINC00265 was positively regulated by expressing *CDK2* (Fig.4B, C). To clarify whether E2F1 can promote *CDK2* transcription, we used E2F1 specific antibody to conduct CHIP-qPCR in Hep3B and BEL-7402 cells. Findings demonstrated that there was a remarkable

enrichment of *CDK2* promoter sequence in the protein-DNA complex containing E2F1 (Fig.4D). In addition, LINC00265 overexpression in HCC cells enhanced binding between E2F1 and *CDK2* (Fig.4E). Next, the binding sequence was truncated or mutated, and dual-luciferase reporter gene assay highlighted that site 3 was the specific site for the binding relationship between E2F1 and *CDK2* promoter region (Fig.4F, G). Western blot and qRT-PCR authenticated that, in Hep3B cells, LINC00265 overexpression could markedly increase *CDK2* expression, while knocking-down of E2F1 counteracted promoting effects of LINC00265 overexpression on *CDK2* expression (Fig.4H, I), in BEL-7402 cells, LINC00265 depletion repressed *CDK2* expression, which was partly reversed by E2F1 overexpression. These data suggested that LINC00265 positively regulated *CDK2* expression via modulating E2F1.

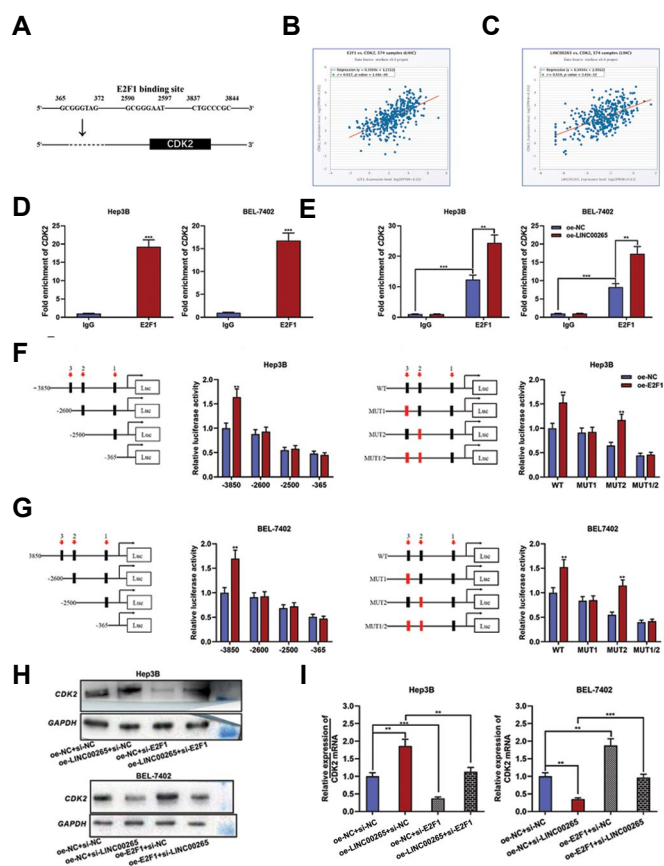


Fig.4: Effects of LINC00265/E2F1 on the expression level of *CDK2* in HCC cells. **A.** PROMO databases were used to predict the binding sites between E2F1 and *CDK2* promoter sequences. **B, C.** Correlations between E2F1 and *CDK2* expressions, LINC00265 and *CDK2* expressions were analyzed by the StarBase database. **D.** Binding relationship between E2F1 and *CDK2* promoter region in Hep3B and BEL-7402 cells was detected with ChIP-qPCR assay. **E.** Effects of LINC00265 on the binding between E2F1 and *CDK2* in Hep3B and BEL-7402 cells were detected by ChIP-qPCR assay. **F.** Dual-luciferase reporter assay was used to detect the specific binding sites between E2F1 and the *CDK2* promoter region in Hep3B cells (3, 2 and 1 respectively indicated site 3, site 2 and site 1). **G.** Dual-luciferase reporter assay was used to detect the specific binding sites between E2F1 and the *CDK2* promoter region in BEL-7402 cells (3, 2 and 1 respectively indicated site 3, site 2 and site 1). **H, I.** Western blot and qRT-PCR were used to detect regulatory effects of LINC00265 and E2F1 on *CDK2* expression in Hep3B and BEL-7402 cells. All experiments were repeated three times with three replicates for each repeat. HCC; Hepatocellular carcinoma, ChIP-qPCR; Chromatin immunoprecipitation (ChIP)-quantitative polymerase chain reaction (qPCR), **, $P < 0.01$, and ***, $P < 0.001$.

LINC00265 regulated proliferation, migration and invasion of HCC cells through the E2F1/*CDK2* axis

To verify the regulatory function of LINC00265 on proliferation, migration and invasion of HCC cells through E2F1/*CDK2* axis, we transfected LINC00265 overexpression plasmids, si-E2F1 and si-*CDK2* into Hep3B cells and si-LINC00265#1. Moreover, we transfected E2F1 overexpression plasmids and *CDK2* overexpression plasmids into BEL-7402 cells. Western blot and qRT-PCR proved that transfection was successful (Fig.5A, B). CCK-8, EdU and Transwell assays showed that LINC00265 overexpression could dramatically promote proliferation, migration and invasion of Hep3B cells, while knocking-down of E2F1 or *CDK2* could partially weaken these effects. Besides, knocking-down of LINC00265 could significantly inhibit malignant biological behaviors of BEL-7402 cells, while E2F1 or *CDK2* overexpression could partially reverse these effects (Fig.5C-F). These results further validated that biological function of LINC00265 in HCC cells were at least partly dependent on the E2F1/*CDK2* axis.

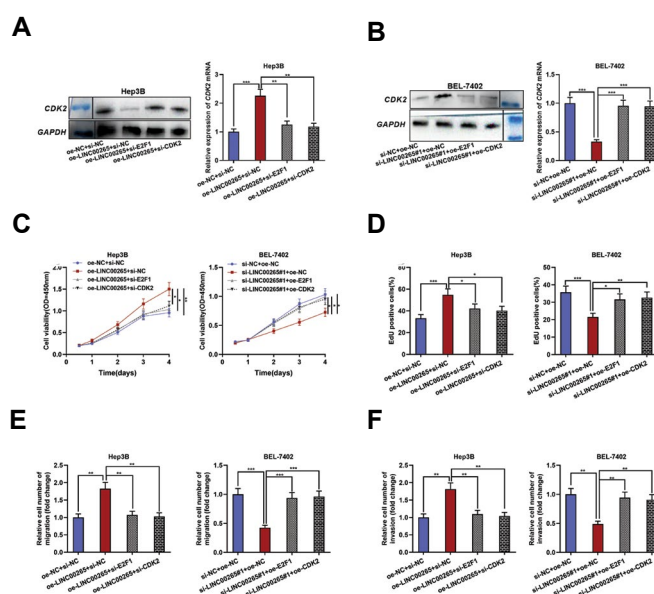


Fig.5: LINC00265 regulates proliferation, migration and invasion of HCC cells via E2F1/*CDK2* axis. **A, B.** LINC00265 overexpression plasmid, si-E2F1 or si-*CDK2* was transfected into Hep3B cells; si-LINC00265#1, E2F1 overexpression plasmid or *CDK2* overexpression plasmid was transfected into BEL-7402 cells. Transfection efficiency was detected by Western blot and qRT-PCR. **C, D.** After transfection, CCK-8 and EdU assays were used to detect proliferation of BEL-7402 and Hep3B cells. **E, F.** After transfection, migration and invasion of BEL-7402 and Hep3B cells were detected by Transwell assay. All experiments were repeated three times with three replicates for each repeat. HCC; Hepatocellular carcinoma, qRT-PCR; Quantitative real-time polymerase chain reaction, *, $P < 0.05$, **, $P < 0.01$, and ***, $P < 0.001$.

Discussion

lncRNAs cannot encode protein due to the lack of open reading frame (16). lncRNAs were initially considered as “transcriptional noise”. However, recent studies report that lncRNAs are crucial regulators in both physiological and pathological processes (17-19). Dysfunction of lncRNA features prominently in the pathogenesis of HCC (20).

LINC00265 expression is up-regulated in osteosarcoma, which is associated with the poor prognosis of patients; overexpression of LINC00265 promotes proliferation, migration, invasion and angiogenesis of osteosarcoma cells via up-regulating vav guanine nucleotide exchange factor 3 (*VAV3*) (21). Additionally, LINC00265 was highly expressed in the bone marrow and serum of patients with acute myeloid leukemia; LINC00265 overexpression could promote malignant biological behaviors of leukemia cells via activating the PI3K/AKT signaling pathway (22). In colorectal cancer, overexpression of LINC00265 recruited USP7 enzyme by up-regulating expression of *ZMIZ2*. Ubiquitination of USP7 enzyme activated β -catenin pathway, thus promoting progression of CRC (23). Furthermore, LINC00265 knock-down can inhibit gastric cancer cell proliferation *in vitro* (24). However, the role of LINC00265 in HCC remained unclear. In the present study, we reported for the first time that LINC00265 expression was elevated in HCC. This was significantly correlated with the unfavorable prognosis of HCC patients. Functionally, LINC00265 overexpression could markedly promote proliferation, migration and invasion of HCC cells, while knocking-down LINC00265 had opposite effects. These demonstrations suggested that LINC00265 was a potential biomarker and therapy target for HCC.

E2F1 is a member of the E2F family of cell cycle-related transcription factors (25). Reportedly, E2F1 functions as an oncogene in cancer biology (26). For example, E2F1 induces transcription of cell division cycle associated 5, thus activating AKT signaling pathway, promoting HCC cell proliferation and inhibiting apoptosis (27). Knocking-down of E2F1 can partially abolish promoting effects of SIRT5 on growth and invasion of HCC cells (28); E2F1 overexpression promoted transcription of *DEAD/H-box helicase 11* and activated PI3K/AKT/mTOR signaling pathway. This promoted the malignant biological behaviors of HCC cells (29). Previous studies reported that some lncRNAs can participate in regulating cancer progression via modulating the function of E2F1. Specifically, lncRNA DLX5-AS1 promoted transcription of *DLX6* by recruiting E2F1 to the promoter region of *DLX6*, and potentiated proliferation and invasion of endometrial cancer cells (30). In this study, we found that LINC00265 could interact with E2F1 and LINC00265 overexpression promoted *CDK2* transcription by recruiting E2F1 to the promoter region of *CDK2*. Our data suggested that LINC00265 is a novel regulator of the E2F1.

In eukaryotic cells, cyclin-dependent kinase (CDK) regulates initiation and progression of cell cycle, proliferation and apoptosis of cells (30, 31). *CDK2* consists of 298 amino acid residues and it can interact with different substances to regulate cell cycle progression (32). Besides, *CDK2* is associated with progression of various cancers (12, 33). For example, *CDK2* expression is up-regulated in gastric cancer, and depletion of *CDK2* expression inhibits the aerobic glycolysis of gastric cancer cells and promotes expression of tumor suppressor *SIRT5*

(34); *HOXA7* facilitates HCC progression via regulating *cyclin E1/CDK2* (35); suppressing *S100P* expression triggers down-regulation of *CDK2* expression, thus inhibiting mitosis of HCC cells (36). In this study, we found that overexpression of LINC00265 could enhance binding of E2F1 to the promoter region of *CDK2*. Thus this up-regulates *CDK2* expression and promotes proliferation, migration and invasion of HCC cells. These data partly explained mechanism of *CDK2* dysregulation in HCC.

Conclusion

In this study, we substantiated that LINC00265 expression is highly expressed in HCC, implying poor prognosis of HCC patients. We also demonstrated that LINC00265 regulated E2F1/*CDK2* axis, thereby promoting HCC cell proliferation, migration and invasion. Altogether, our data suggested that LINC00265 may act as a new screening biomarker and potential therapy target for HCC patients.

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Authors' Contributions

Q.Ch.; Designed the experiments, the structure and the logical flow of the whole manuscript. B.G., X.Zh., W.Zh.; Conducted the most *in vitro* experiments. Y.M., Zh.S.; Collected the tissue samples. G.X.; Helped with literature reviewing and cell culture. All authors participated in the manuscript drafting. B.G., X.Zh., W.Zh., Q.Ch.; Are the main contributors. B.G., Q.Ch.; Reviewed the completed manuscript and made the final revisions. All authors read and approved the final manuscript.

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