European Review for Medical and Pharmacological Sciences 2021; 25: 6208-6219

LINC00205, a YY1-modulated lncRNA, serves as a sponge for miR-26a-5p facilitating the proliferation of hepatocellular carcinoma cells by elevating CDK6

T. CHENG[1], Y. YAO[1], S. ZHANG[1], X.-N. ZHANG[1], A.-H. ZHANG[1], W. YANG[2], C.-Z. HOU[1]

[1]Department of General Surgery, Xi'an Children's Hospital, Xi'an, China
[2]Department of Hepatobiliary Surgery, The First Affiliated Hospital of Xi'an Jiaotong University, Xi'an, China

Abstract. – OBJECTIVE: LINC00205, a bidirectional lncRNA, located at human chromosome 21q22.3, was recently characterized as an oncogenic molecule contributing to cell proliferation in several cancers, including hepatocellular carcinoma (HCC). In the present study, we aim to probe the new molecular mechanism for LINC00205 controlling the proliferation of HCC cells.

PATIENTS AND METHODS: The expression status of LINC00205, miR-26a-5p, as well as CDK6 in HCC tissues/cell lines was determined by quantitative real-time PCR (qPCR). The cell proliferative activity was measured by using the Cell Counting Kit (CCK)-8 assay. Flow cytometry was performed to analyze cell cycle progression and apoptosis induction. The interaction among LINC00205, miR-26a-5p and CDK6, as well as transcription efficiency of LINC00205 promoter were examined by Dual-Luciferase reporter assay. Western blot was conducted to evaluate the protein levels of CDK6 in SNU-449 cells. The direct interplay between YY1 and LINC00205 promoter was detected by ChIP-qPCR.

RESULTS: LINC00205 was strongly expressed in HCC tissues and cell lines. Elevated LINC00205 expression was positively associated with worse prognosis as well as pathological grade in HCC. Suppression of LINC00205 could impede the proliferation of HCC cells by triggering the G0/G1-phase cell cycle arrest and apoptosis *in vitro*. Mechanistically, we illustrated that LINC00205 could accelerate the proliferation of HCC cells by boosting CDK6 expression via sponging miR-26a-5p. Moreover, we unveiled that LINC00205 could be activated by transcription factor Yin Yang-1 (YY1) as its direct downstream target.

CONCLUSIONS: LINC00205, a novel YY1-modulated lncRNA, can facilitate the proliferation of HCC cells through YY1/miR-26a-5p/CDK6 pathway, and may serve as a promising diagnostic biomarker and therapeutic target for HCC.

Key Words:
Hepatocellular carcinoma, LINC00205, Cell proliferation, MiR-26a-5p, CDK6, YY1.

Introduction

Hepatocellular carcinoma (HCC), as the major histological subtype of primary liver cancer, is the seventh-most common malignant tumor type, as well as the second foremost reason for cancer-related death[1]. Numerous etiological factors are considered to be closely associated with HCC development, such as chronic infections with HBV and HCV, aflatoxin exposure, excessive alcohol intake and cigarette smoking[2]. HCC remains a global health challenge and its incidence is growing worldwide, especially in Asia and Africa[3]. Most HCC patients have poor outcomes due to the lack of effective early diagnosis and suitable treatment options. Despite remarkable progress has been achieved in treatment strategies, such as surgical treatment (liver resection and liver transplantation), ablation treatment (radiofrequency and microwave), radiotherapy, chemotherapy, as well as immunotherapy, the prognosis of HCC patients is yet undesirable due to the insidious nature and high rates of metastasis and recurrence[4,5]. Hence, elucidating the molecular mechanisms underlying HCC progression might be conducive to develop more effective diagnostic biomarkers and therapeutic targets.

Corresponding Author: Chongzhi Hou, MD; e-mail: chongzhihou@126.com

Long non-coding RNAs (lncRNAs), as a novel kind of regulatory RNA molecules, are widely involved in cancer progression[6]. For HCC, many HCC-related lncRNAs have been identified, which are abnormally expressed in HCC and can modulate tumor growth, invasion, metastasis, as well as angiogenesis by interplaying with various types of biomolecules, involving mRNA, microRNA (miRNA), protein and DNA (promoter)[7,8]. For example, He et al[9] manifested ZFPM2-AS1, an activated lncRNA in HCC, was remarkably related to poor survival and could promote cell proliferation, migration and invasion by sponging miR-139 and raising the expression level of GDF10, which encodes a secreted ligand of the TGF-beta superfamily of proteins.

Long intergenic non-protein-coding RNA 00205 (LINC00205), a lncRNA situated on human chromosome 21q22.3, shares a bidirectional promoter with protein O-fucosyltransferase 2 (POFUT2). LINC00205, as an oncogenic molecule, was remarkably boosted in several tumor types and closely related to prognosis and tumor progression. For instance, in lung cancer, suppression of LINC00205 could weaken cell proliferation, as well as migration, and aggravate cell apoptosis via recruiting FUS to sustain the mRNA stability of CSDE1[10]; in retinoblastoma (RB), LINC00205 was proven to promote the proliferation, migration and invasion of RB cells through increasing HMGB1 expression via sponging miR-665[11]. For HCC, a study conducted by Zhang et al[12] demonstrated LINC00205 was elevated in HCC and tightly associated with tumor size, venous infiltration and advanced tumor stages, knockdown of LINC00205 could dramatically suppress the capacity of cell growth, migration and invasion via sponging miR-122-5p. Contradictorily, another research performed by Long et al[13] found that LINC00205 may play a cancer suppressive role through interplaying with miR-184 to elevate the expression level of EPHX1, which was down-regulated in HCC, and could retard cell proliferation, migration, as well as cell cycle progression and induce apoptosis.

The current study was designed to explore the functions of LINC00205 on cell proliferation and its new regulatory mechanism in HCC. Our results disclosed that YY1/LINC00205/miR-26a-5p/CDK6 axis, as a new modulation pathway, contributed to the HCC cell proliferation and might serve as a promising diagnostic biomarker and therapeutic target for HCC.

Patients and Methods

Tissue Specimens

Eighteen pairs of HCC samples and matched nonneoplastic hepatic tissues were gathered from patients in the First Affiliated Hospital of Xi'an Jiaotong University (Xi'an, Shaanxi Province, China). Tissue samples were quenched in liquid nitrogen immediately after excision. Consent was acquired from all patients and was approved by the Ethics Committee of the First Affiliated Hospital of Xi'an Jiaotong University (No. 2017-145).

Cell Culture and Transfection

Human hepatocellular carcinoma cells (HepG2, SNU-475, SNU-449, Huh-7, SK-Hep-1), as well as a human liver epithelial cell line THLE-3 were gathered from the American Type Culture Collection (ATCC, Manassas, VA, USA). All cells were cultured using Dulbecco's Modified Eagle Medium (DMEM; Gibco, Rockville, MD, USA) supplemented with 10% FBS and 1× Penicillin-Streptomycin (100 U/ml-100 μg/ml, Sigma-Aldrich, St. Louis, MO, USA) in incubator (37°C, 5% CO_2).

Short interfering RNAs (siRNA) targeting LINC00205 and negative control (NC) were produced by Synbio Technologies (Suzhou, Jiangsu Province, China) as follows: siLINC00205, 5'-GAAGCAGAAAUCCAUAGAACA-3'; siNC, 5'-GCGUUGCUGUCGCUCUAUUCU-3'. Predesigned siRNAs for CDK6 (sc-29264) and YY1 (sc-36863) were obtained from Santa Cruz Biotechnology (Santa Cruz, CA, USA). MiR-26a-5p mimics/inhibitors and its biotin labeling were synthesized by GenePharma (Shanghai, China). The LINC00205-overexpressing vector (pcDNA-LINC00205), YY1-overexpressing vector (pcDNA-YY1), as well as pcDNA3.1(+) empty control vector was also synthesized from GenePharma (Shanghai, China). siRNA, miRNA mimics/inhibitors and vectors mentioned above were transfected into cells by HighGene plus Transfection reagent (ABclonal, Wuhan, Hubei Province, China).

RNA Isolation and Quantitative Real-Time PCR (qPCR)

Total RNA was separated by Beyozol (Beyotime, Shanghai, China). cDNA templates for qPCR were synthesized by miScript II RT Kit (Qiagen, Hilden, Germany) with total RNA. qPCR was conducted with UltraSYBR Mixture (CWBio, Beijing, China). The relative expres-

sion level of target genes was calculated using the $2^{-\Delta\Delta Ct}$ method. β-actin and U6 were used as an endogenous control for lncRNA/mRNA and miRNA evaluation, separately. Primers were showed as follows: LINC00205 (forward: 5'-TGAATCAGTACACGCCCACC-3', reverse: 5'-TACTGGGCAAAAGATGCGGT-3'), CDK6 (forward: 5'-GGATAAAGTTCCAGAGCCTGGAG-3', reverse: 5'-GCGATGCACTACTCGGTGTGAA-3'), YY1 (forward: 5'-GGAGGAATACCTGGCATTGACC-3', reverse: 5'-CCCTGAACATCTTTGTGCAGCC-3'), β-actin (forward: 5'- CCTGGCACCCAGCACAAT -3', reverse: 5'-GCCGATCCACACGGAGTACT -3'), miR-26a-5p (forward: 5'-TTCAAGTAATCCAGGATAGGCTAAA-3'), U6 (forward: 5'-CTCGCTTCGGCAGCACATATACT-3', reverse : 5'-ACGCTTCACGAATTTGCGTGTC-3') .

Cell Proliferation Assay

The cell proliferation capacity was detected using cell counting kit-8 (CCK-8) assay. In brief, cells (4×10^3 cells/well) were inoculated into 96-well plates and then incubated with 10 μL CCK-8 solution (Beyotime, Shanghai, China) for 1 hours 30 minutes in incubator (37°C, 5% CO_2). The absorbance at a wavelength of 450 nm was determined by microplate reader.

Cell Cycle and Apoptosis Detection by Flow Cytometry

Regarding cell cycle analysis, the cells were collected by trypsinization and fixed with 70% ice-cold ethanol overnight. Next, the fixed cells were incubated with propidium iodide (PI)/RNase A staining solution (Beyotime, Shanghai, China) at room temperature for 25 minutes, and the cell cycle arrest was evaluated by flow cytometry.

Regarding apoptosis detection, the cells were collected by trypsinization. Next, the cells were stained with FITC-Annexin V and propidium iodide (PI) and examined by flow cytometry according to the manufacturer's instructions for FTIC Annexin V Apoptosis Detection Kit I (BD Biosciences, San Diego, CA, USA).

Dual-Luciferase Reporter Assay

To assess the binding relationship between miR-26a-5p and LINC00205 or CDK6, the wild-type (wt) sequence of LINC00205 or CDK6 and the corresponding sequence harboring mutant (mut) miR-26a-5p targeting site was integrated into the psiCHECK-2 reporter vector (Promega, Madison, WI, USA) by GenePharma (Shanghai, China). Then, the reporter plasmid and miR-26a-5p mimics or pcDNA-LINC00205 vector were co-transfected into HEK293 cells by HighGene plus Transfection reagent (ABclonal, Wuhan, Hubei Province, China), and the Luciferase activity was detected.

To evaluate the promoter activity, the DNA sequence of LINC00205 promoter (2000 bp upstream from TSS) was cloned using the forward primer (GTGAAGGCAGTGAGTGGCCTGT) and reverse primer (AGACTCTGCGCACGCGCAG), and integrated into pGL3-Basic vector (Promega, Madison, WI, USA). Subsequently, reporter plasmid and pcDNA-YY1 vector were co-transfected into HEK293 cells, and the Luciferase activity was then detected.

The Luciferase activity was determined using the TransDetect Dual-Luciferase Reporter Assay Kit (Transgen, Beijing, China).

RNA Pull Down

The biotinylated-hsa-miR-26a-5p mimics and the negative control cel-miR-239b mimics (GenePharma, Shanghai, China) were transfected into SNU-449 and SK-Hep-1 cells for 36 hours. Then, the whole-cell lysates were prepared in RIP buffer (150 mM KCl, 25 mM Tris pH 7.4, 5 mM EDTA, 0.5 mM DTT, 0.5% NP40, 100 U/ml RNAase inhibitor and protease inhibitors). RNA-RNA complexes were pulled down by incubation with Streptavidin Magnetic Beads (NEB, S1420S) overnight at 4°C, and were then isolated using Quick-RNA™ Miniprep Plus Kit (Zymo Research, Irvine, CA, USA). The enrichment status of LINC00205 and CDK6 was examined by qPCR.

Western Blot

Total proteins were isolated by Radio Immunoprecipitation Assay (RIPA) Lysis Buffer containing broad-spectrum protease inhibitors (Boster, Wuhan, Hubei Province, China), and separated by 10% Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis (SDS-PAGE). The protein was transferred onto polyvinylidene difluoride (PVDF) membrane (0.45 μm) by wet transfer system, and was then blocked with bovine serum albumin (BSA) Blocking Buffer (CWBio, Beijing, China) for 1 hour at room temperature. After incubating with the primary antibodies and horse radish peroxidase (HRP)-conjugated the secondary antibodies, the blots were detected using enhanced chemiluminescence (ECL) Western

Blot Kit (CWBio, Beijing, China). The antibodies used for Western blot were as follows: anti-CDK6 (13331T, CST; 1:1000), anti-β-ACTIN (PA1-183, Invitrogen; 1:1000), HRP-conjugated Affinipure Goat Anti-Rabbit IgG (H+L) (SA00001-2, Proteintech; 1:10000). β-Actin was used as an internal control.

Chromatin Immunoprecipitation

Chromatin immunoprecipitation (ChIP) experiment was carried out using the EpiTect ChIP OneDay Kit (Qiagen, Hilden, Germany; # 334471). The antibodies used for immunoprecipitating protein-DNA complexes were as follows: rabbit anti-YY1 (ab12132, Abcam, Cambridge, MA, USA), and normal rabbit IgG (ab171870, Abcam). The immunoprecipitated DNA was extracted and detected by qPCR experiment. Three pairs of primers adopted for examining LINC00205 promoter were as follow: Primer 1 (-3 to -212 bp), 5'-CCAGCAGCAGGAAGACGAAG-3' (forward) and 5'-ACTCTGCGCACGCGCAGTT-3' (reverse); Primer 2 (-354 to -487 bp), 5'-CCTGCCCCATAACTCCGCT-3' (forward) and 5'-GCAGGGTCAGAGGATGGGATG-3' (reverse); Primer 3 (-469 to -636 bp), 5'-CCCATCCCATCCCTCCTGG-3' (forward) and 5'-AGCGGAGTTATGGGGCAGG-3' (reverse).

Bioinformatics Analysis

The expression status of LINC00205 in HCC (n=374) and non-tumor tissues (n=50) were analyzed by ENCORI (http://starbase.sysu.edu.cn/panCancer.php). The relationship between LINC00205 expression and overall survival, as well as disease free survival of patients was assessed by a web tool GEPIA (http://gepia.cancer-pku.cn/) based on TCGA data. The association between LINC00205 expression and pathological stage of patients was also evaluated using GEPIA.

Statistical Analysis

The data were presented as mean ± SD. Student's *t*-test was adopted to assess two-group comparisons. One-way ANOVA with Tukey's test was used to assess statistically significant differences among multi-group. Spearman's correlation was used to evaluate the correlation between the expression of two molecules. A p-value < 0.05 was considered to be statistically significant. All statistical analysis was carried out using SPSS 23.0 (IBM, Armonk, NY, USA).

Results

Boosted LINC00205 was Related to Unfavorable Prognosis in HCC

First, we evaluate the expression status of LINC00205 and its clinical significance in HCC by bioinformatics analysis, the ENCORI results displayed LINC00205 was highly expressed in HCC (Figure 1A), the GEPIA results disclosed that up-regulation of LINC00205 was significantly correlated with shorter overall survival and disease free survival in HCC patients (Figure 1B and C), and was positively related to advanced pathological stage (Figure 1D). Second, overexpression of LINC00205 was verified in 18 pairs of HCC and matched nonneoplastic hepatic tissues by qPCR. Unsurprisingly, the expression level of LINC00205 was dramatically elevated in HCC tissue compared with normal samples (Figure 1E). Additionally, we also demonstrated that the expression level of LINC00205 was increased in all five examined HCC cell lines (HepG2, SNU-475, SNU-449, Huh-7, SK-Hep-1) when compared with THLE-3 cells, a human liver epithelial cell line (Figure 1F).

Suppression of LINC00205 Impeded the Proliferation of HCC Cells by Triggering the G0/G1-Phase Cell Cycle Arrest and Accelerate Apoptosis

To ascertain the function of LINC00205 on the proliferation of HCC cells, the knockdown experiment was conducted using siRNA to explore the impacts of LINC00205 suppression on the proliferation, cell cycle progression and apoptosis in both SNU-449 and SK-Hep-1 cells that strongly expressed LINC00205. Knockdown efficiency of siRNA targeting LINC00205 in both SUN-449 and SK-Hep-1 cells was evaluated by qPCR (Figure 2A). CCK-8 assay exhibited LINC00205 suppression could restrain the proliferation capacity of both SUN-449 as well as SK-Hep-1 cells (Figure 2B). To probe whether LINC00205 suppression affects the cell proliferation by modulating cell cycle and apoptosis, flow cytometric analysis was performed to detect cell cycle and apoptosis after cells were stained with PI or/and Annexin V, and the results showed LINC00205 suppression could trigger the G0/G1-phase cell cycle arrest (Figure 2C) and accelerate apoptosis (Figure 2D) in SUN-449 and SK-Hep-1. Taken together, our results indicated that blocking of LINC00205

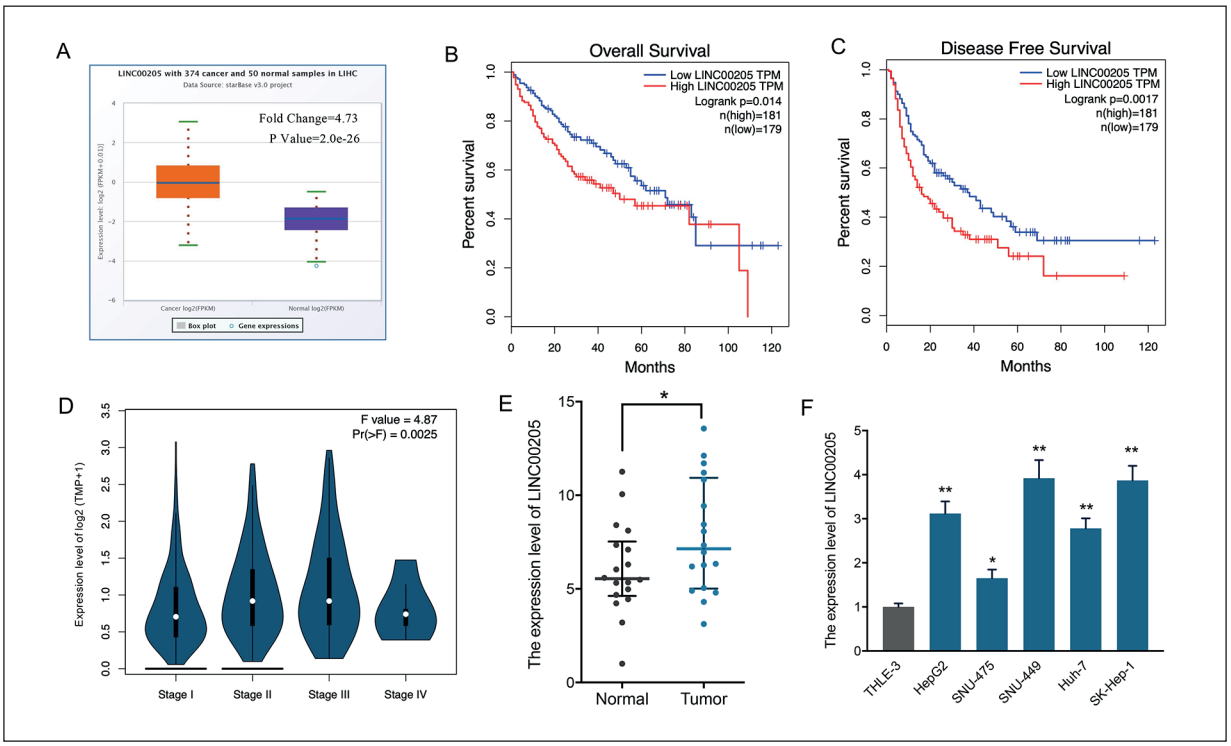

Figure 1. Elevated LINC00205 is related to unfavorable prognosis in hepatocellular carcinoma (HCC). **A**, The expression status of LINC00205 in HCC (n=374) and non-tumorous tissues (n=50) tissues were evaluated by ENCORI. **B**, The association between LINC00205 expression with overall survival was evaluated using GEPIA. **C**, The association between LINC00205 expression with disease free survival was evaluated by GEPIA. **D**, The association between LINC00205 expression with pathological stage was evaluated by GEPIA. **E**, The expression status of LINC00205 was validated in human HCC and non-tumorous tissues (n = 18) by qPCR. **F**, The expression status of LINC00205 in HCC cell lines was examined by qPCR. * $p < 0.05$, ** $p < 0.01$.

could restrain the proliferation of HCC cell by inducing the G0/G1-phase cell cycle arrest as well as apoptosis.

LINC00205 Acts as the Sponge for MiR-26a-5p in HCC Cells

LINC00205 has been illustrated to facilitate tumor development as a ceRNA to modulating tumor-associated genes through sponging miRNAs in HCC[11-13]. Consequently, we conjectured that LINC00205 might control the cell proliferation of HCC through ceRNA mechanism that sponging other miRNAs as well as downstream targets. The Ago-CLIP-seq data was used to examine the miRNAs interacting with LINC00205 by the online tool ENCORI, and we found LINC00205 harbored 91 miRNAs binding sites (**Supplementary Table I**), including miR-665, miR-122-5p and miR-184, which have been previously reported that can be sponged by LINC00205[11-13]. In the current study, we chose the miR-26a-5p as the research object because it has been found to be low expressed in HCC and serve as a tumor suppressor via interplaying with lncRNA[14,15], as well as circRNA[16]. The binding relationship between miR-26a-5p and LINC00205 was exhibited in Figure 3A. Here, we uncovered that miR-26a-5p was decreased in HCC tissues and cell lines (Figure 3B and C), and its expression was negatively related to LINC00205 expression in HCC tissues (Figure 3D). Dual-Luciferase reporter assays showed that miR-26a-5p mimics could dramatically hamper the Luciferase activities of vector harboring LINC00205-wt sequence rather than mutant type (Figure 3E). Furthermore, biotin-labelled miRNA pull-down experiment demonstrated that LINC00205 was remarkably enriched in the biotin-conjugated miR-26a-5p-arrested complexes compared to that in the negative control cel-miR-239b (Figure 3F). Finally, we also demonstrated the expression level of miR-26a-5p could be elevated through silencing LINC00205 (Figure 3G), whereas LINC00205 expression could be restrained after transfecting

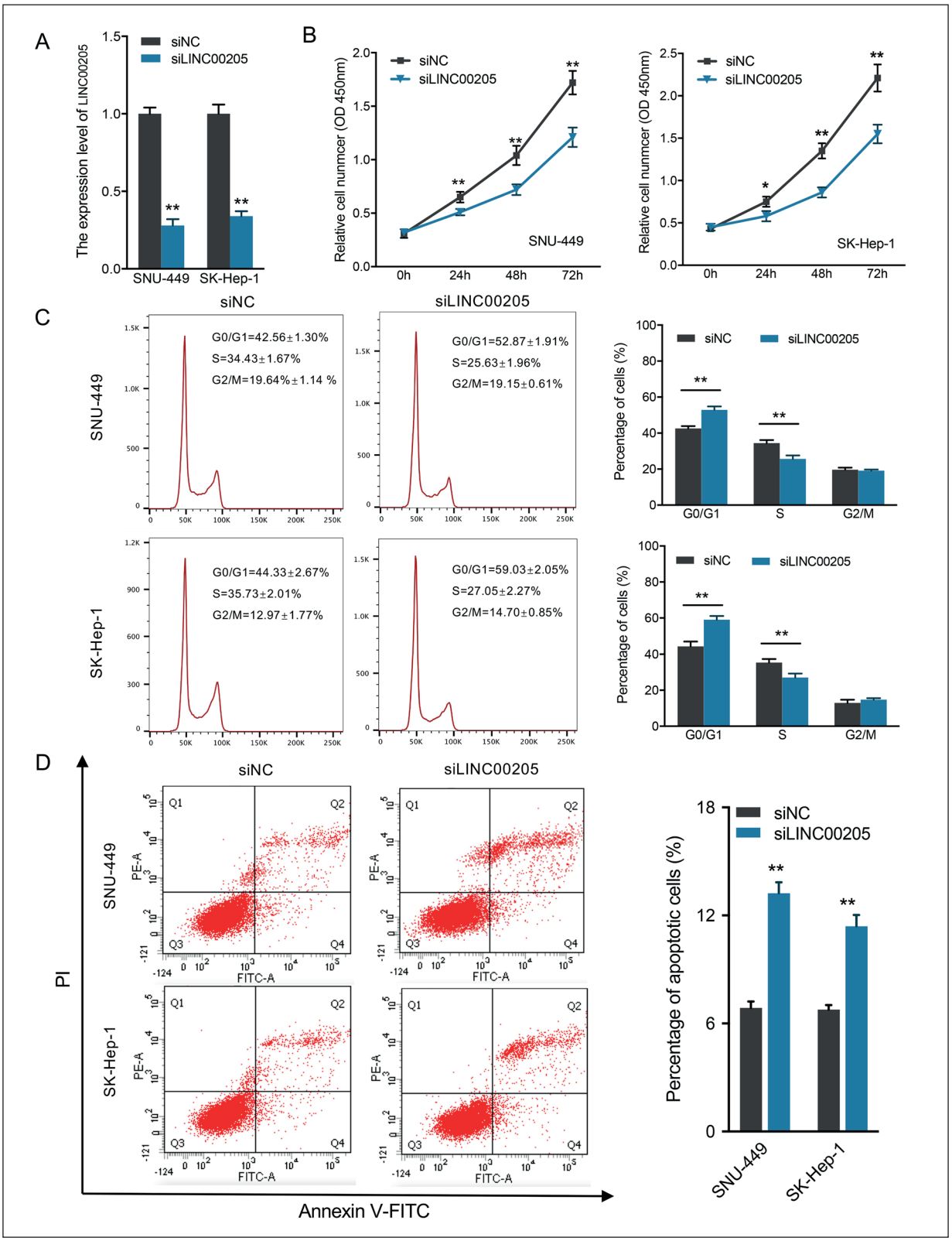

Figure 2. Downregulation of LINC00205 inhibits HCC cell proliferation. **A**, The silencing efficiency of siRNA targeting LINC00205 in SNU-449 and SK-Hep-1 cells was confirmed by qPCR. **B**, The impact of LINC00205 downregulation on the proliferative capacity of HCC cells was determined using CCK-8 assay. **C** and **D**, The impact of LINC00205 downregulation on cell cycle progression and apoptosis was detected by flow cytometry. * $p < 0.05$, ** $p < 0.01$.

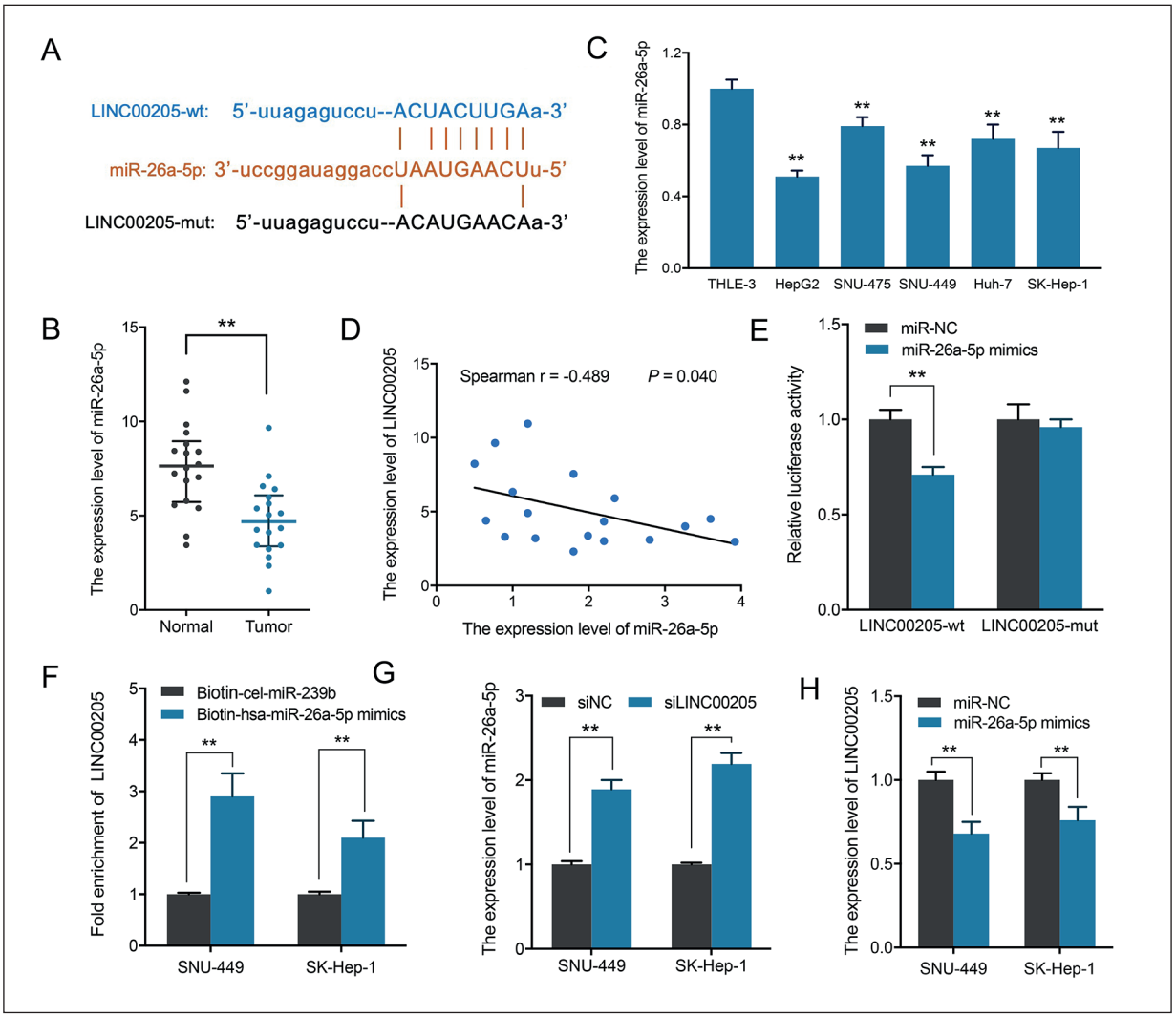

Figure 3. LINC00205 sponged miR-26a-5p in HCC cells. **A**, The putative miR-26a-5p binding sites harboring by LINC00205 wild type (LINC00205-wt) and its mutation (LINC00205-mut) were shown. **B**, The expression status of miR-26a-5p in HCC and non-tumorous tissues (n = 18) was detected by qPCR . **C**, The expression status of miR-26a-5p in HCC cell lines was evaluated by qPCR. **D**, The interrelation of LINC00205 expression with miR-26a-5p was assessed by Spearman's coefficient. **E**, The interaction between LINC00205 and miR-26a-5p was examined by Dual-Luciferase reporter assays. **F**, The interaction between LINC00205 and miR-26a-5p was validated by RNA pull-down with biotin-conjugated miR-26a-5p or negative control cel-miR-239b. **G**, The impact of LINC00205 downregulation on the expression status of miR-26a-5p in HCC cells. **H**, The impact of miR-26a-5p upregulation on the expression status of LINC00205 in HCC cells. * $p < 0.05$, ** $p < 0.01$.

miR-26a-5p mimics (Figure 3H). In summary, the above results indicated that LINC00205 could serve as the sponge for miR-26a-5p in HCC cells.

LINC00205 Controls the Proliferation of HCC Cells via Elevating CDK6 Through Sponging MiR-26a-5p

To elaborate the molecular mechanism underlying LINC00205 modulating the proliferation of HCC through sponging miR-26a-5p, we attempted to screen and characterize downstream targets of miR-26a-5p using the online tool ENCORI and TargetScan, and we found cyclin dependent kinase 6 (CDK6), a crucial modulator of cell cycle and cell growth, existed a potential targeting relationship with miR-26a-5p (Figure 4A). CDK6 expression was validated by qPCR, and the results indicated that CDK6 was highly expressed in HCC tissues as well as cell lines (Figure 4B and C), and the expression of CDK6 was positively related with LINC00205 in HCC tissues (Figure 4D). Biotin-labelled miRNA pull-down experiment revealed that

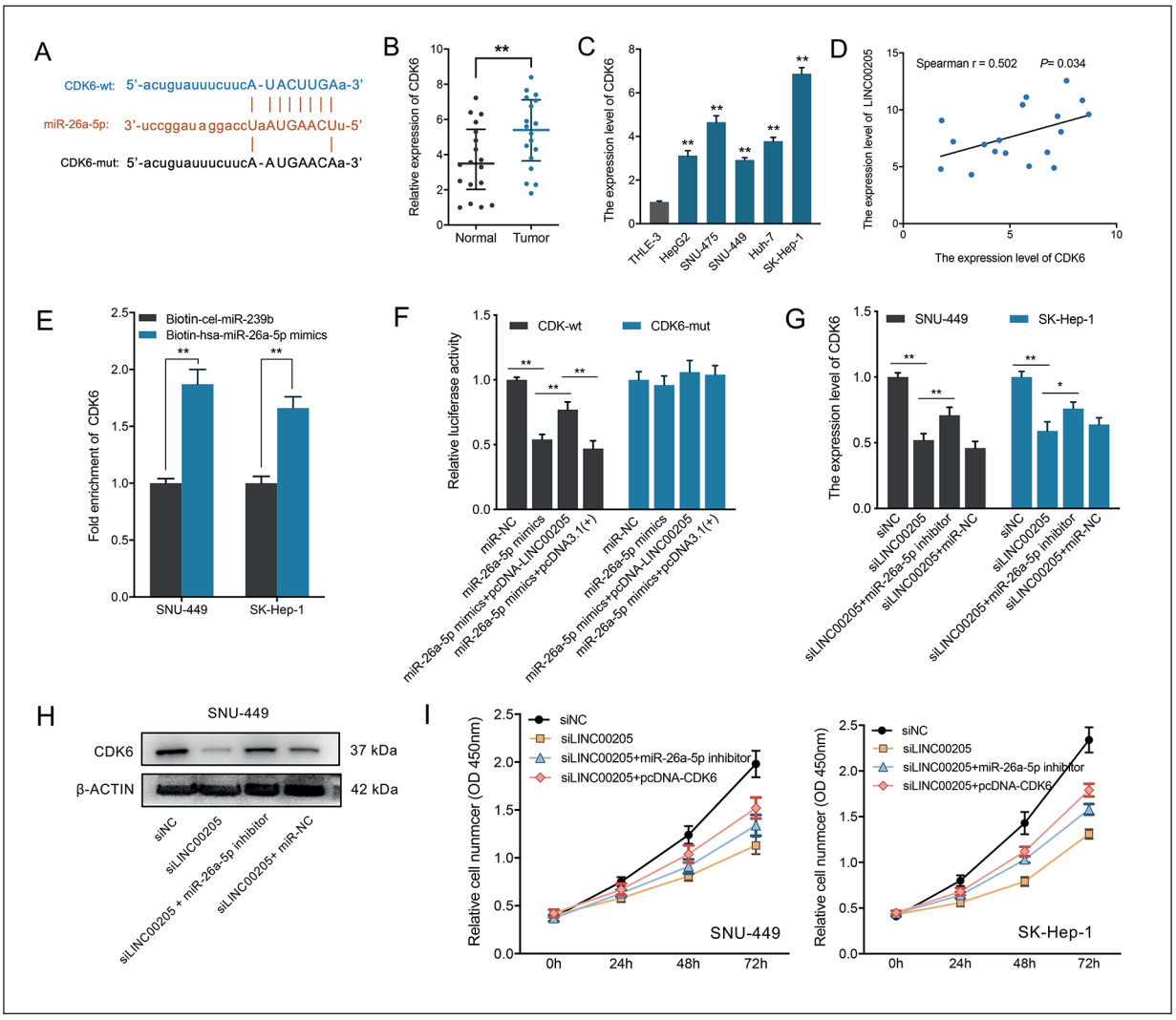

Figure 4. LINC00205 modulates the proliferation of HCC cells via triggering miR-26a-5p/CDK6 pathway. **A**, The putative miR-26a-5p binding sites harboring by CDK6 wild type (CDK6-wt) and its mutation (CDK6-mut) were shown. **B**, The expression status of CDK6 in HCC and non-tumorous tissues (n = 18) was assessed by qPCR. **C**, The expression status of CDK6 in HCC cell lines was examined by qPCR. **D**, The interrelation of LINC00205 expression with CDK6 was assessed by Spearman's coefficient. **E**, The interaction between CDK6 and miR-26a-5p was detected by RNA pull-down with biotin-conjugated miR-26a-5p or negative control cel-miR-239b. **F**, The interaction between CDK6 and miR-26a-5p, and the impact of LINC00205 upregulation on its interplay was confirmed by Dual-Luciferase reporter assays. **G**, LINC00205 modulated the CDK6 transcripts by miR-26a-5p. **H**, LINC00205 modulated the CDK6 protein level by miR-26a-5p in SNU-449 cells. **I**, The rescue experiment displayed that LINC00205 downregulation could restrain the HCC cell proliferation via miR-26a-5p/CDK6 pathway. * $p < 0.05$, ** $p < 0.01$.

CDK6 mRNA could be dramatically trapped by biotin-conjugated miR-26a-5p-mimics rather than the negative control (Figure 4E). Dual-Luciferase reporter assays demonstrated that the activity of reporter harboring wild-type sequence of CDK6 rather than mutation site was remarkably inhibited by miR-26a-5p mimics and could be restored upon up-regulation of LINC00205, suggesting there was a direct interaction among LINC00205, miR-26a-5p and CDK6 (Figure 4F). Furthermore, the modulating role of LINC00205 and miR-26a-5p on CDK6 mRNA level was first confirmed by qPCR, and we revealed that suppressing LINC00205 could decline the CDK6 expression in both SUN-449 and SK-Hep-1 cells, meanwhile the reduction of CDK6 caused by LINC00205 silencing could be recovered by miR-26a-5p inhibitor (Figure 4G). Then the western blot was conducted to verify

the modulating effect of LINC00205 and miR-26a-5p on the CDK6 protein in SUN-449 cells, we disclosed that the trends in the alterations at protein level were consistent with the results of qPCR (Figure 4H). Additionally, we also uncovered that suppression of miR-26a-5p or ectopic expression of CDK6 could reverse the inhibition of cell proliferation that triggered by LINC00205 silencing in both SUN-449 and SK-Hep-1 cells (Figure 4I). Altogether, the above results manifest that LINC00205 could control the proliferation of HCC cells by elevating CDK6 via sponging miR-26a-5p.

LINC00205 can be Activated by the Transcription Factor YY1 in HCC Cells

To probe the reason why LINC00205 was overexpressed in HCC, we checked transcription factor binding sites harbored by the LINC00205 promoter using ChIP-seq data of HepG2 cells on UCSC Genome Browser (http://genome.ucsc.edu), and the Yin Yang-1 (YY1) binding site was found near the upstream of the transcription start site (TSS) of LINC00205 (Figure 5A). We further analyzed the LINC00205 promoter region (2000 bp up-stream of TSS) using transcription factor searching tools of JASPAR (http://jaspar.genereg.

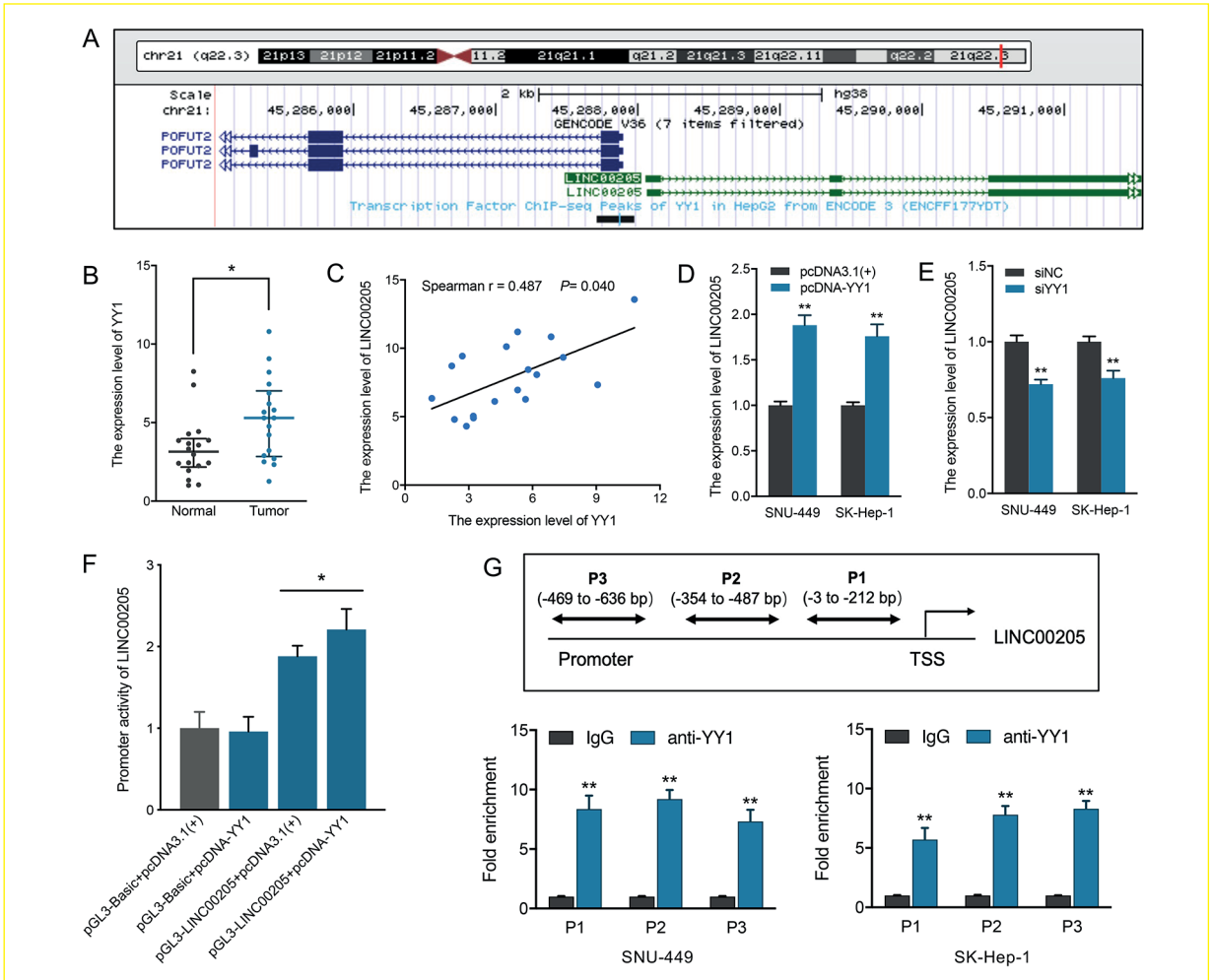

Figure 5. LINC00205 can be activated by the transcription factor YY1. **A**, YY1 binding status of LINC00205 promoter was checked using ChIP-seq data of HepG2 cells by UCSC Genome Browser. **B**, The expression status of YY1 in HCC and non-tumorous tissues (n = 18) was detected by qPCR. **C**, The interrelation of LINC00205 expression with YY1 was assessed by Spearman's coefficient. **D**, The impact of YY1 upregulation on the LINC00205 expression in HCC cells was examined by qPCR. **E**, The impact of YY1 downregulation on the LINC00205 expression in HCC cells was assessed by qPCR. **F**, The impact of YY1 upregulation on the promoter activities of LINC00205 was determined by Dual-Luciferase reporter assays. **G**, The interaction between YY1 and LINC00205 promoter was detected in SNU-449 and SK-Hep-1 cells by ChIP-qPCR, 1% of chromatin was used as input DNA control. * $p < 0.05$, ** $p < 0.01$.

netl), and 9 potential YY1 binding sites were obtained (**Supplementary Table II**). Intriguingly, YY1 have been demonstrated to be elevated in HCC and can aggravate the HCC progression by suppressing HCC differentiation and activating MYC signaling[17,18]. In the current research, we also disclosed that the YY1 expression was remarkably boosted in HCC tissues (Figure 5B), and positively associated with LINC00205 expression (Figure 5C). Subsequently, we examined the modulation roles of YY1 on LINC00205 expression in HCC cells by up-regulating or down-regulating YY1, and qPCR results showed that up-regulation of YY1 could elevate the expression level of LINC00205 in both SUN-449 and SK-Hep-1 cells (Figure 5D), while silencing of YY1 restrained LINC00205 expression (Figure 5E). To verify LINC00205 can be transcriptionally activated by YY1, LINC00205 promoter were integrated into pGL3-basic plasmid and co-transfected with YY1 expression plasmid into HEK-293T cells, and we discovered promoter activity of LINC00205 could be strengthened by overexpressing YY1 (Figure 5F). In addition, the direct interaction between YY1 and LINC00205 promoter was identified using ChIP-qPCR, and we found YY1 was significantly enriched on LINC00205 promoter in both SUN-449 and SK-Hep-1 cells (Figure 5G). Taken together, above results displayed that the up-regulation of LINC00205 in HCC could be attributed to the transcription factor YY1.

Discussion

Growing evidence uncovered that lncRNAs, as a new sort of modulators for gene expression, exert crucial functions in tumor development, including HCC[19,20]. Until now, a large stash of aberrantly expressed lncRNAs has been characterized to be involved in HCC, such as HOTAIR[21], HEIH[22], MVIH[23], ATB[24], and so on. Recently, LINC00205, a bidirectional lncRNA, situated at human chromosome 21q22.3, has been revealed to be activated in some cancers and can promote tumor advancement, involving lung cancer[10], retinoblastoma[11], as well as hepatocellular carcinoma[12]. Yet, the potential molecular mechanism of LINC00205 promoting the HCC progression is substantially unclear, the current study was conducted to survey the novel molecular mechanism by which LINC00205 regulates the proliferation of HCC cells.

First of all, we disclosed LINC00205 was dramatically upregulated in HCC tissues and cell lines, and positively related to unfavorable prognosis as well as pathological stage, indicating LINC00205 might be a promising biomarker for early diagnosis and prognosis evaluation of HCC. Consistent with our findings, the study conducted by Zhang et al[12] also revealed that LINC00205 was dramatically elevated in both HCC tissues and cell lines, and its boosted expression was significantly related to tumor size, venous infiltration and tumor stages.

Second, we illustrated that silencing LINC00205 could restrain the proliferation of SUN-449 and SK-Hep-1 cells, and our results were consistent with previous findings in other HCC cell lines. Zhang et al[12] uncovered LINC00205 knockdown could suppress the proliferation of Hep3B and Huh-7 cells. Remarkably, in our study, it was revealed for the first time that knockdown of LINC00205 can induce the G0/G1 phase cell cycle block and apoptosis. Xie et al[10] regarding lung cancer revealed that silencing LINC00205 could also inhibit lung cancer cell growth thought aggravating apoptosis.

Thirdly, a new regulatory pathway launched by LINC00205 was demonstrated to be involved in the modulation of HCC cell proliferation, that is LINC00205 could boosting CDK6 by arresting miR-26a-5p (i.e., miR-26a-5p/CDK6 axis). Multiple ceRNA regulatory axes participated by LINC00205 have been discovered to be involved in cancer progression, such as miR-665/HMGB1 in retinoblastoma[11]. Here, we revealed LINC00205 as a sponge could directly interplay with miR-26a-5p in HCC cells. Precedent studies have disclosed miR-26a-5p was lowly expressed in HCC, and its down-regulation was positively correlated with worse prognosis in HCC patients[25, 26]. Notably, miR-26a-5p could suppress the HCC cell proliferation through modulating some critical oncogenes, such as HMGA2[26], DNMT3B[27], AURKA[28], GSK3β[14], implying that miR-26a-5p is likely to exert a tumor suppressive role in HCC. In the present study, we demonstrated CDK6 as a new target of miR-26a-5p could contribute to modulation of HCC cell proliferation launched by LINC00205 via constituting the ceRNA pathway. CDK6, an oncogenic molecule, has been disclosed to exert vital functions on tumor advancement in several cancer types, including HCC[29]. Interestingly, several recent studies found CDK6 could facilitate the HCC development via constituting lncRNA-miRNA-mRNA regulation

network. For instance, lncRNA CRNDE has been shown to regulate cell growth and metastasis via miR-33a-5p/CDK6 axis[30]; lncRNA NNT-AS1 was uncovered to modulate the proliferation of HCC cells by miR-363/CDK6 pathway[31]. In the present paper, CDK6 was disclosed to be activated in HCC and could affect the proliferation of HCC cells as downstream target of LINC00205/ miR-26a-5p pathway.

Finally, we illuminated that LINC00205 transcription could be triggered by a transcription factor YY1 in HCC. YY1, a ubiquitously expressed zinc finger transcription factor that contains four C2H2 zinc finger domains, exerts crucial functions in multiple cellular processes, involving DNA replication, cell multiplication, apoptosis, autophagy land chromatin modeling[32]. Rong et al[33] found YY1 could directly interact with the promoter of lncRNA LINC01134 and enhance its transcription, while LINC01134 is an oncogenic lncRNA and contributes to HCC progression by affecting cell growth, metastasis and EMT via miR-324-5p/IGF2BP1 pathway. In the current paper, LINC00205 was characterized as a new target of transcription factor YY1.

Conclusions

The present study uncovered that LINC00205, a YY1-modulated lncRNA, was overexpressed in HCC, and facilitated the proliferation of HCC cells through triggering cell cycle and apoptosis via a new ceRNA axis of miR-26a-5p/CDK6, indicating that LINC00205 might serve as a potential diagnostic marker and therapeutic target for HCC.

Conflict of Interest

The Authors declare that they have no conflict of interests.

Acknowledgements

This work was supported by the Key Research and Development Program of Shaanxi Province of China (2018KW-062).

References

1) McGlynn KA, Petrick JL, El-Serag HB. Epidemiology of hepatocellular carcinoma. Hepatology 2021; 73 Suppl 1: 4-13.
2) Mohammadian M, Mahdavifar N, Mohammadian-Hafshejani A, Salehiniya H. Liver cancer in the world: epidemiology, incidence, mortality and risk factors. World Cancer Research Journal 2018; 5: e1082.
3) Llovet JM, Kelley RK, Villanueva A, Singal AG, Pikarsky E, Roayaie S, Lencioni R, Koike K, Zucman-Rossi J, Finn RS. Hepatocellular carcinoma. Nat Rev Dis Primers 2021; 7: 6.
4) Koulouris A, Tsagkaris C, Spyrou V, Pappa E, Troullinou A, Nikolaou M. Hepatocellular carcinoma: an overview of the changing landscape of treatment options. J Hepatocell Carcinoma 2021; 8: 387-401.
5) Kole C, Charalampakis N, Tsakatikas S, Vailas M, Moris D, Gkotsis E, Kykalos S, Karamouzis MV, Schizas D. Immunotherapy for hepatocellular carcinoma: a 2021 update. Cancers (Basel) 2020; 12: 2859.
6) Goodall GJ, Wickramasinghe VO. RNA in cancer. Nat Rev Cancer 2021; 21: 22-36.
7) Xie C, Li SY, Fang JH, Zhu Y, Yang JE. Functional long non-coding RNAs in hepatocellular carcinoma. Cancer Lett 2021; 500: 281-291.
8) Niu ZS, Wang WH, Dong XN, Tian LM. Role of long noncoding RNA-mediated competing endogenous RNA regulatory network in hepatocellular carcinoma. World J Gastroenterol 2020; 26: 4240-4260.
9) He H, Wang Y, Ye P, Yi D, Cheng Y, Tang H, Zhu Z, Wang X, Jin S. Long noncoding RNA ZFPM2-AS1 acts as a miRNA sponge and promotes cell invasion through regulation of miR-139/GDF10 in hepatocellular carcinoma. J Exp Clin Cancer Res 2020; 39: 159.
10) Xie P, Guo Y. LINC00205 promotes malignancy in lung cancer by recruiting FUS and stabilizing CSDE1. Biosci Rep 2020; 40: BSR20190701.
11) Zhang S, Long J, Hu Y. Long noncoding RNA LINC00205 enhances the malignant characteristics of retinoblastoma by acting as a molecular sponge of microRNA-665 and consequently increasing HMGB1 expression. Biochem Biophys Res Commun 2020; 526: 396-403.
12) Zhang L, Wang Y, Sun J, Ma H, Guo C. LINC00205 promotes proliferation, migration and invasion of HCC cells by targeting miR-122-5p. Pathol Res Pract 2019; 215: 152515.
13) Long X, Li Q, Zhi LJ, Li JM, Wang ZY. LINC00205 modulates the expression of EPHX1 through the inhibition of miR-184 in hepatocellular carcinoma as a ceRNA. J Cell Physiol 2020; 235: 3013-3021.
14) Li Y, Guo D, Zhao Y, Ren M, Lu G, Wang Y, Zhang J, Mi C, He S, Lu X. Long non-coding RNA SNHG5 promotes human hepatocellular carcinoma progression by regulating miR-26a-5p/GSK3beta signal pathway. Cell Death Dis 2018; 9: 888.
15) Li ML, Zhang Y, Ma LT. LncRNA HCG11 accelerates the progression of hepatocellular carcinoma via miR-26a-5p/ATG12 axis. Eur Rev Med Pharmacol Sci 2019; 23: 10708-10720.
16) Yang L, Xue H, Sun Y, Zhang L, Xue F, Ge R. CircularRNA-9119 protects hepatocellular carcinoma cells from apoptosis by intercepting miR-26a/

JAK1/STAT3 signaling. Cell Death Dis 2020; 11: 605.

17) Zhang S, Jiang T, Feng L, Sun J, Lu H, Wang Q, Pan M, Huang D, Wang X, Wang L, Jin H. Yin Yang-1 suppresses differentiation of hepatocellular carcinoma cells through the downregulation of CCAAT/enhancer-binding protein alpha. J Mol Med (Berl) 2012; 90: 1069-1077.

18) Mei C, Jiang X, Gu Y, Wu X, Ma W, Chen X, Wang G, Yao Y, Liu Y, Zhang Z, Yuan Y. YY1-mediated reticulocalbin-2 upregulation promotes the hepatocellular carcinoma progression via activating MYC signaling. Am J Cancer Res 2021; 11: 2238-2251.

19) Huang Z, Zhou JK, Peng Y, He W, Huang C. The role of long noncoding RNAs in hepatocellular carcinoma. Mol Cancer 2020; 19: 77.

20) Zhu X, Pan H, Liu L. Long noncoding RNA network: Novel insight into hepatocellular carcinoma metastasis (Review). Int J Mol Med 2021; 48: 134.

21) Liu Z, Ouyang G, Lu W, Zhang H. Long non-coding RNA HOTAIR promotes hepatocellular carcinoma progression by regulating miR-526b-3p/DHX33 axis. Genes Genomics 2021; 43: 857-868.

22) Yang F, Zhang L, Huo XS, Yuan JH, Xu D, Yuan SX, Zhu N, Zhou WP, Yang GS, Wang YZ, Shang JL, Gao CF, Zhang FR, Wang F, Sun SH. Long noncoding RNA high expression in hepatocellular carcinoma facilitates tumor growth through enhancer of zeste homolog 2 in humans. Hepatology 2011; 54: 1679-1689.

23) Yuan SX, Yang F, Yang Y, Tao QF, Zhang J, Huang G, Yang Y, Wang RY, Yang S, Huo XS, Zhang L, Wang F, Sun SH, Zhou WP. Long noncoding RNA associated with microvascular invasion in hepatocellular carcinoma promotes angiogenesis and serves as a predictor for hepatocellular carcinoma patients' poor recurrence-free survival after hepatectomy. Hepatology 2012; 56: 2231-2241.

24) Yuan JH, Yang F, Wang F, Ma JZ, Guo YJ, Tao QF, Liu F, Pan W, Wang TT, Zhou CC, Wang SB, Wang YZ, Yang Y, Yang N, Zhou WP, Yang GS, Sun SH. A long noncoding RNA activated by TGF-beta promotes the invasion-metastasis cascade in hepatocellular carcinoma. Cancer Cell 2014; 25: 666-681.

25) Chang L, Li K, Guo T. MiR-26a-5p suppresses tumor metastasis by regulating EMT and is associated with prognosis in HCC. Clin Transl Oncol 2017; 19: 695-703.

26) Zhu WJ, Yan Y, Zhang JW, Tang YD, Han B. Effect and mechanism of miR-26a-5p on proliferation and apoptosis of hepatocellular carcinoma cells. Cancer Manag Res 2020; 12: 3013-3022.

27) Li Y, Ren M, Zhao Y, Lu X, Wang M, Hu J, Lu G, He S. MicroRNA-26a inhibits proliferation and metastasis of human hepatocellular carcinoma by regulating DNMT3B-MEG3 axis. Oncol Rep 2017; 37: 3527-3535.

28) Yuan YL, Yu H, Mu SM, Dong YD, Li Y. MiR-26a-5p inhibits cell proliferation and enhances doxorubicin sensitivity in HCC cells via targeting AURKA. Technol Cancer Res Treat 2019; 18: 1533033819851833.

29) Tadesse S, Yu M, Kumarasiri M, Le BT, Wang S. Targeting CDK6 in cancer: state of the art and new insights. Cell Cycle 2015; 14: 3220-3230.

30) Lin C, Xiang Y, Sheng J, Liu S, Cui M, Zhang X. Long non-coding RNA CRNDE promotes malignant progression of hepatocellular carcinoma through the miR-33a-5p/CDK6 axis. J Physiol Biochem 2020; 76: 469-481.

31) Lu YB, Jiang Q, Yang MY, Zhou JX, Zhang Q. Long noncoding RNA NNT-AS1 promotes hepatocellular carcinoma progression and metastasis through miR-363/CDK6 axis. Oncotarget 2017; 8: 88804-88814.

32) Verheul TCJ, van Hijfte L, Perenthaler E, Barakat TS. The why of YY1: mechanisms of transcriptional regulation by Yin Yang 1. Front Cell Dev Biol 2020; 8: 592164.

33) Rong Z, Wang Z, Wang X, Qin C, Geng W. Molecular interplay between linc01134 and YY1 dictates hepatocellular carcinoma progression. J Exp Clin Cancer Res 2020; 39: 61.