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Piplartine suppresses proliferation and invasion of hepatocellular carcinoma by LINC01391-modulated Wnt/β-catenin pathway inactivation through ICAT

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ABSTRACT

Although piplartine is regarded as an anticancer agent, the relationship between long noncoding RNAs (lncRNAs), which are involved in various diseases (e.g., tumors) and piplartine in hepatocellular carcinoma (HCC) remains unclear. We identified LINC01391 using microarray analysis and validated its expression by qRT-PCR. Functional assays were applied to evaluate the biological effects of LINC01391 and inhibitory of β-catenin and T-cell factor (ICAT) on HepG2 and SMMC-7721 cells. The binding relationship between LINC01391 and ICAT was determined by RNA pull-down and RNA immunoprecipitation (RIP). Results showed that piplartine attenuated cell proliferation and invasion but promoted cell apoptosis. Upregulation of LINC01391 induced by piplartine inhibited HCC cell proliferation, invasion in vitro, and tumor growth in vivo. LINC01391 interacted with ICAT and promoted its inhibitory effect on the Wnt/β-catenin pathway, as enhanced interaction between β-catenin and ICAT, and dampened interaction of β-catenin and TCF/LEF were induced by overexpression of LINC01391. Knockdown of ICAT also promoted cell proliferation in vitro and tumor growth in vivo. Our study supported a role for piplartine and LINC01391 in HCC treatment. We found that LINC01391 inhibited the Wnt/β-catenin pathway and suppressed tumor growth via ICAT.

1. Introduction

Hepatocellular carcinoma (HCC) is one of the most lethal malignant tumors and remains the third leading cause of cancer-related deaths worldwide [1,2]. Although considerable advances have been made in medical therapy and surgical treatments, the morbidity and mortality of HCC remain high [3]. Approximately 696,000 patients die of liver cancer every year. East Asia, especially China, is the most prevalent region of HCC, accounting for more than half of the cases worldwide [4,5]. Therefore, understanding the pathophysiological mechanism underlying the occurrence and development of HCC and developing potential therapeutic targets are necessary.

Piplartine, a biologically active alkaloid extracted from the roots of long pepper, may have anti-depressive [6], heart-protecting, anti-inflammatory [7], anti-atherosclerosis [8] and a variety of pharmacological activities. Recently, piplartine was shown to kill tumor cells selectively without affecting normal cells [7–9]. This selective anti-tumor property is related to the two key enzymes regulating ROS, GSTP1, and CBR1, thereby inducing the accumulation of reactive oxygen species in cancer cells [10,11]. Previous studies demonstrated the cytotoxic effect of piplartine and regarded it as a potential antitumor drug [12–14]. The anticancer properties of piplartine were evaluated in HCC [15].

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However, the mechanism underlying the effects of piplartine on HCC remains unclear.

Long noncoding RNAs (lncRNAs) are transcripts longer than 200 nucleotides, which do not encode a protein or encodes a short peptide [16,17]. LncRNAs were originally regarded as transcriptional noises that did not exert any biological functions [18]. Recently, accumulated studies have demonstrated that lncRNAs are related to tumors. Dysregulation of lncRNAs regulated a variety of biological functions, such as apoptosis, colonization, migration, and invasion [19]. In addition, lncRNAs play a pivotal role in mRNA stability, protein translation, and chromatin dynamics [20,21].

In the present study, we adopted a comprehensive analysis to investigate the differences between lncRNAs expression profiles in hepatocellular cancer cells with or without piplartine using lncRNA microarray. LINC01391 is a long intergenic non-protein coding RNA, and the length of LINC01391 is 3538 bp which have no structure available. LINC01391 was identified as a candidate therapeutic target for HCC. Then, we systematically evaluated the functions of LINC01391 by cell and animal models. The effects of LINC01391 on cell proliferation, cell cycle distribution, cell invasion, and apoptosis were explored. A number of signaling pathways, including Wnt/β-catenin signaling pathways, were involved in HCC [22]. Here, we found that LINC01391 attenuated the Wnt/β-catenin pathway by binding and promoting the expression of inhibitor of β-catenin and T-cell factor (ICAT) in HCC cells.

2. Materials and methods

2.1. Cell culture and infection

HepG2 and SMMC-7721 cell lines were obtained from the ATCC (Manassas, VA, USA) and maintained in Dulbecco's Modified Eagle's Medium (DMEM) that contained 10% fetal bovine serum, 100 U/mL penicillin, and 100 μg/mL streptomycin. For transfections, cells at the confluence of 60–80% were infected with 1×10^6 recombinant lentivirus-transducing units and 6 μg/mL Polybrene (Sigma). Stably transfected cells were selected by 2 μg/mL puromycin treatment for 2 weeks. Stably transfected cells were picked via flow cytometry for subsequent assays. Plasmid and lentivirus used in this study were purchased from GenePharma, Shanghai, China.

2.2. Piplartine treatment

HepG2 and SMMC-7721 cells were pre-seeded in a six-well plate. A total of 10 μM piplartin was added into the culture medium at the indicated time. Then, cells were collected for subsequent assays.

2.3. Cell proliferation assays

We used the CCK-8 assay and EDU assay to measure cell proliferation ability. For CCK-8 assay, 1×10^3 cells seeded in a 96-well plate overnight. Then, the culture medium was replaced with a complete medium that contained a 10 μL CCK-8 solution. The spectrophotometric absorbance at 450 nm was determined after 2 h. For the EDU assay, the Cell-Light EdU Apollo 567 In-Vitro Imaging Kit (RiboBio) conducted following manufacturer's instructions. Briefly, 1×10^3 were seeded in a 96-well plate and cultured with 50 μM EdU for 2 h at 37 °C. After being fixed with paraformaldehyde, cells were stained and observed under a fluorescence microscope (20×). Five random fields were captured to calculate the EdU-positive cells.

2.4. Flow cytometry analysis

For cell apoptosis and cell cycle detection, HCC cells with indicated treatment were stained with annexin V and propidium iodide using an Apoptosis Detection Kit (Beyotime, Nantong, China) following the manufacturer's instructions. The ratio of cell cycle distribution and cell apoptosis between each group were calculated and compared.

2.5. Cell migration and invasion

We used Transwell chambers (Corning, New York, USA) to test cell migration and invasion. In the assay, the negative control of piplartine treatment is culture medium, the negative control of sh-LINC01391 and sh-ICAT treatment is sh-NC and the negative control of LINC01391-overexpressing plasmid treatment is vector. Cells with indicated treatment were suspended in a serum-free medium and seeded into the upper chamber. The complete culture medium was added in the lower chamber as chemokine. After culturing for 48 h, the migrated or invasive cells were stained with crystal violet and observed under a microscope (20×). Evaluation of cell invasion was performed using Transwell chambers pre-coated with matrigel. Five random fields were captured to calculate the cells on the lower membrane surface.

2.6. RNA extraction and microarray analysis

We extracted total RNA from tissues or cells using Trizol reagent (Invitrogen, Carlsbad, CA) following the manufacturer's protocol. Then, nanoDropND-2000 was applied to quantify the extracted RNA. The RNA from HepG2 cells with or without piplartine treatment was used to synthesize double-stranded cDNA. Then, the cDNA was labeled and hybridized to the LncRNA Expression Microarray (Arraystar, USA) following the manufacturer's protocol. Fluorescence images were obtained by a laser scanner and digitized using Affymetrix Transcriptome Analysis software. The raw data matrix is analyzed by first subtracting the background matrix and then normalization. A 2-sample T-test was used for statistical analysis. The criteria for differently expressed lncRNA was a fold change ≥ 2.0 and a P-value < 0.05.

2.7. qRT-PCR and subcellular fractionation

Quantification expression of LINC01391 and other genes were detected by qRT-PCR following the manufacturer's protocol (Bio-Rad). The Applied Biosystems 7500 Sequence Detection system was used to conduct qRT-PCR analysis. Three replicates were set for each sample. Primer sequences for target genes are listed in Supplementary Table 1. Cytosolic and nuclear fractions were extracted using the Nuclear/Cytoplasmic Isolation Kit (Biovision) following the manufacturer's instructions. Then, we used qRT-PCR to measure the expression of RNU6-1 (nuclear control), GAPDH (cytoplasmic control), and LINC01391 in the fractions.

2.8. Animals and tumor growth

All animal experiments were conducted in accordance with the Guide for the Care and Use of Laboratory Animals with approval of the Animal Ethics Committee of the Lishui Hospital Affiliated by Zhejiang University. Five-week-old pathogen-free nude mice were purchased from the Shanghai Institute for Biological Sciences (SIBS). The animals were housed in laminar airflow cabinets under pathogen-free conditions. Mice were subcutaneously injected into the back of each nude mouse with 1×10^6 cancer cells stably transfected with LINC01391 overexpressing plasmid or scramble vector in a volume of 100 μL Hank's balanced salt solution. The resulting tumor volumes were observed every 7 d. The tumor volume was calculated according to the formula: $V = (\text{length} \times \text{width}^2)/2$. Then, mice were killed to remove and weigh tumors after 5 weeks.

2.9. Immunohistochemical (IHC) analysis

Briefly, the subcutaneous tumor was resected and fixed in 10% formaldehyde. After incubation with anti-Ki-67 antibodies (Abcam)

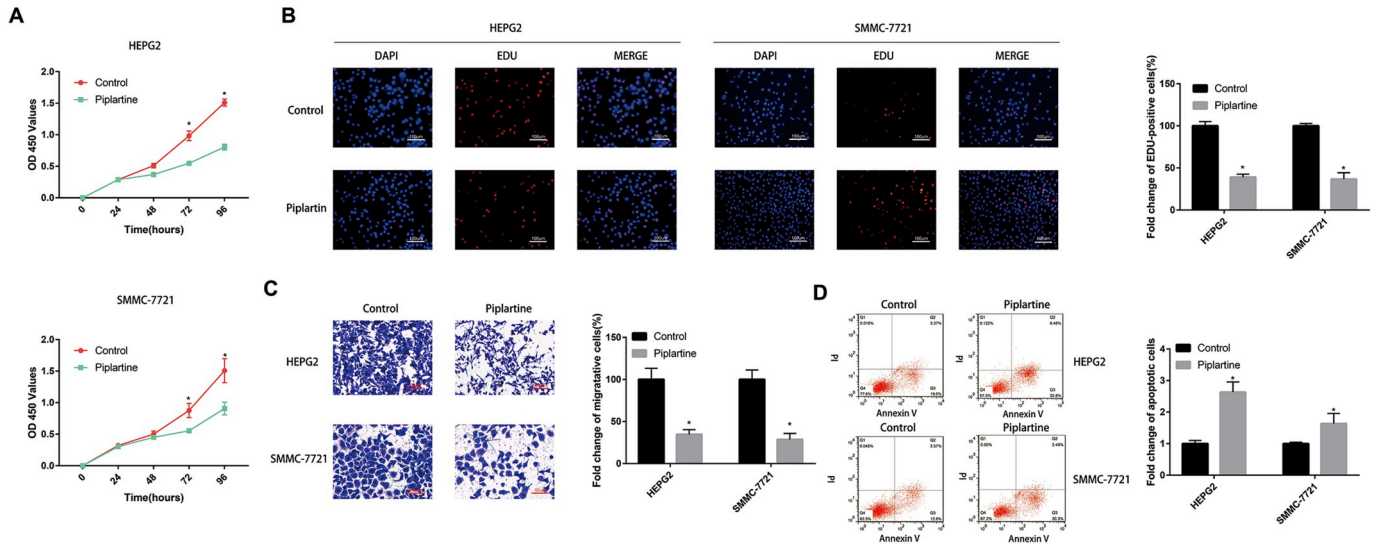

Fig. 1. Piplartine suppressed proliferation and promoted apoptosis of liver cancer cells in vitro. (A) CCK-8 was applied to compare and quantify HepG2 and SMMC-7721 cell proliferation in the presence of 10 μM piplartine at an indicated time. (B) EdU incorporation assay was conducted to determine the effect of 10 μM piplartine on DNA synthesis during cell proliferation. Piplartine decreased the ratio of EdU-positive (S-phase) cells in total cancer cells. (C) Representative images and quantification of the amount of invasive HepG2 and SMMC-7721 cells with or without exogenous piplartine per high-power field. (D) Flow cytometric analysis of the rates of apoptotic HepG2 and SMMC-7721 cells after treatment with 10 μM piplartine. Data are shown as mean ± SEM. *P < 0.05.

overnight, we visualized the slide using streptavidin-horseradish peroxidase-conjugated secondary antibody. Then, hematoxylin staining was conducted on the same slide. Pictures were captured under light microscopy (40×).

2.10. TOPFlash luciferase assay

Wnt/β-catenin TOPFlash reporter (Addgene, 12456) or mutant FOPFlash reporter (Addgene, 12457) were co-transfected with sh-LINC01391, LINC01391, or control vector into HepG2 and SMMC-7721 cells and cultured for 36 h. Then, a dual-detection luciferase detection kit (Promega Corporation) was applied to lyse cells and detect Wnt/β-catenin activation based on the ratio of the fold change of TOPFlash to the FOPFlash control.

2.11. RNA pull-down assay

Briefly, biotin-labeled LINC01391 and its antisense RNAs were transcribed in vitro with the Biotin RNA labeling mix (Roche Diagnostics, USA) and SP6/T7 RNA polymerase (Roche), treated with RNase-free DNase I (Roche) and purified with the RNeasy Mini Kit (Qiagen, USA). Lysates of cancer cell were mixed with biotinylated RNA. Then, the proteins were measured using Western blot analysis.

2.12. Western blot

Whole cell protein lysates were separated on a 10% SDS-PAGE and transferred onto the PVDF membrane and then blocked with 5% non-fat milk. The immune blots were incubated with a primary antibody ICAT (Abcam, 1/1000), TCF3 (Abcam, 1/1000), TCF4 (Abcam, 1/1000), LEF1 (Abcam, 1/1000), β-catenin (Abcam, 1/5000), and GAPDH (Abcam, 1/10000) overnight at 4 °C followed by incubation with secondary antibody HRP-labeled Goat Anti-Rabbit IgG(H + L) (Beyotime, 1/1000) or HRP-labeled Goat Anti-Mouse IgG(H + L) (Beyotime, 1/1000) at room temperature for 2 h. The signals were detected by ChemiDoc MP (Bio-Rad).

2.13. Co-immunoprecipitation and RNA immunoprecipitation

For co-immunoprecipitation, HepG2 with LINC01391 overexpression and negative control cells were crushed in RIPA buffer prior to incubation with β-catenin antibody for 4 h. Then, the precipitate was detected by Western blot. We conducted RIP using a Magna RNA-binding protein immunoprecipitation kit (Millipore, MA) following the manufacturer's recommendation. Briefly, cell lysates were incubated with magnetic beads conjugated with normal human IgG or human anti-ICAT antibody. Then, to isolate the immunoprecipitated RNA, samples were incubated with proteinase K samples. Finally, qRT-PCR was used to quantify the expression of target genes in purified RNAs.

2.14. Statistical analysis

Statistical analysis was conducted using SPSS 20.0 software (Chicago, USA). The results were presented as mean ± SEM. The two-tailed Student's t-test was used to evaluate the data, and $P < 0.05$ was considered significant.

3. Results

3.1. Piplartine impaired cell proliferation, invasion, and enhanced cell apoptosis

We treated HEPG2 and SMMC-7721 with piplartine (0, 5, 10, 15, 20 μM) for 24 h and detected the cell viability. The IC50 of these two cells were near 10 μM (Supplementary Fig. 1(A)). HepG2 and SMMC-7721 cells were exposed to 10 μM piplartine for functional evaluation. The CCK-8 results indicated that piplartine significantly dampened the proliferation of HepG2 and SMMC-7721 cells (Fig. 1(A)). An EDU assay also showed a decreased number of EDU positive HepG2 and SMMC-7721 cells in piplartine treated groups (Fig. 1(B)). To investigate the effect of piplartine on cell migration and invasion, a Transwell assay was conducted. The results demonstrated that piplartine significantly suppressed cell invasion (Fig. 1(C)) in HepG2 and SMMC-7721 cells without affecting cell migration (Supplementary Fig. 1(B)). Flow cytometric analysis showed that the ratio of apoptotic cells was significantly higher in piplartine treated cells than in control cells (Fig. 1(D)). However, we did not observe any difference in cell cycle distribution (Supplementary Fig. 1(C)).

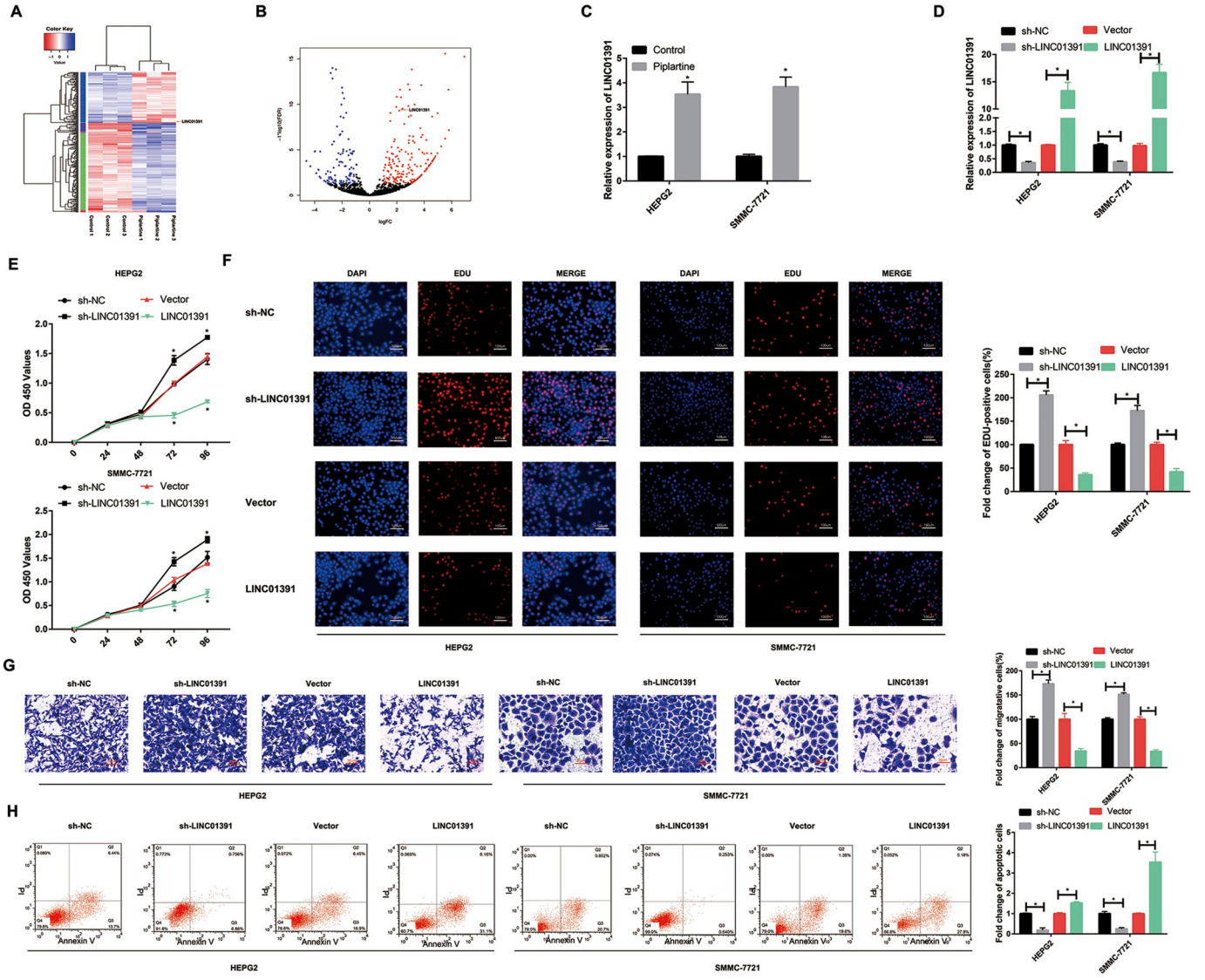

Fig. 2. Downregulation of LINC01391 promoted hepatocellular cancer cells proliferation, invasion, and inhibited apoptosis in vitro. (A) Heatmap and (B) volcano plot illustrating the 294 differentially expressed lncRNAs between piplartine treated HepG2 cells and corresponding negative control HepG2 cells (fold change ≥2, P-value < 0.05). (C) The fold change of LINC01391 expression in HepG2 and SMMC-7721 cells with or without exogenous piplartine. (D) Relative expression levels of LINC01391 expression in HepG2 and SMMC-7721 cells after transfection with sh-LINC01391, LINC01391, and matched control. (E) Assessment of hepatocellular cancer cells growth in vitro using CCK-8 at the indicated time points. (F) Cellular proliferation was estimated from immunofluorescence staining with EdU, and the percentage of EdU-positive nuclei was quantified. (G) Transwell invasion assay in sh-LINC01391, LINC01391, and matched control transfected HepG2 and SMMC-7721 cells was shown. (H) Flow cytometric analysis of the rates of apoptotic HepG2 and SMMC-7721 cells after knockdown or overexpression of LINC01391. Data are shown as the mean ± SEM. *P < 0.05.

3.2. LINC01391 inhibited cell proliferation and invasion

To identify lncRNAs associated with piplartine, we analyzed the lncRNA expression profile from three pairs of HepG2 with or without treatment with piplartine by microarray analysis. In total, we found 294 dysregulated lncRNAs including 188 upregulated lncRNAs in piplartine-treated HepG2 cell (Fig. 2(A) and (B)). Most of these lncRNAs were novel lncRNAs. We focused mainly on the upregulated lncRNAs because they can be readily used as therapeutic targets, compared with the downregulated lncRNAs. In particular, we found that the LINC01391 were remarkably increased in the piplartine-treated group. To validate our microarray findings, expressions of LINC01391 in HepG2 and SMMC-7721 cells with piplartine treatment were measured by qRT-PCR. qRT-PCR analysis confirmed the microarray findings, thereby demonstrating that piplartine promoted the expression of LINC01391 (Fig. 2(C)).

To explore the function of LINC01391, we adopted short hairpin RNA (shRNA) and plasmid to exogenously knockdown or overexpress the expression of LINC01391 in both HepG2 and SMMC-7721 cells (Fig. 2(D)). CCK-8 and EDU assays showed that the downregulation of LINC01391 expression significantly promoted cell proliferation, whereas LINC01391 overexpression did the opposite (Fig. 2(E) and (F)). Transwell assays demonstrated that knockdown of LINC01391 markedly enhanced cell invasion compared with the negative control, whereas enforced LINC01391 expression had an opposite effect (Fig. 2(G)). To clarify the effect of LINC01391 on cell apoptosis, we used flow cytometry. A decrease in the fraction of apoptotic cells was observed in LINC01391 knockdown cells, whereas overexpressed LINC01391 promoted cell apoptosis (Fig. 2(H)).

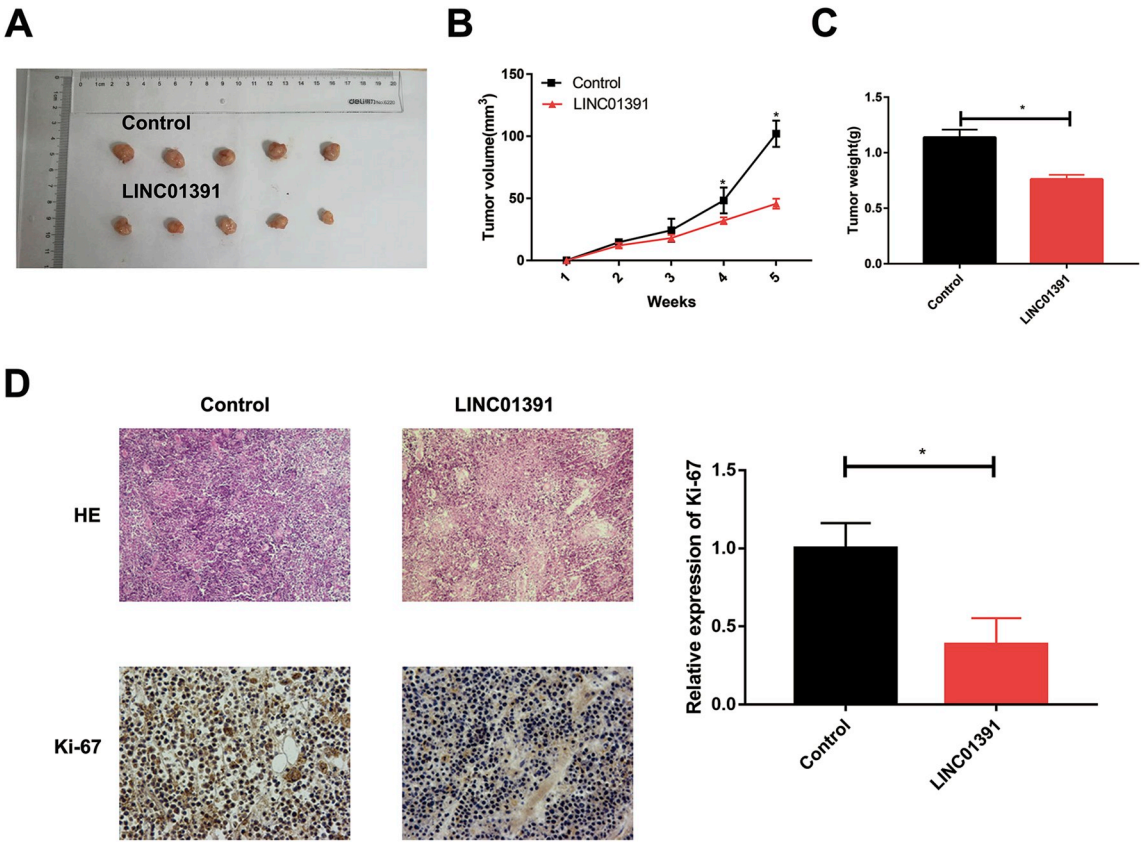

Fig. 3. LINC01391 suppressed the growth of hepatocellular cancer cells in vivo. LINC01391-overexpressing virus treated HepG2 cells were injected subcutaneously into the back of nude mice (1×10^6 cells per mouse, five mice per group). (A) Tumors collected from mice were exhibited. (B) Tumor volume curve of mouse upon LINC01391-overexpressing plasmid or vector treatment was analyzed. (C) Tumor weight of mouse was measured. (D) Representative images of H&E staining (upper) and immunohistochemical staining of tumor specimens for Ki-67 (lower) in the indicated groups (Ki-67: a cellular marker for proliferation). Data are shown as the mean ± SEM. *P < 0.05.

LINC01391 impaired tumor growth in vivo

To validate the effect of LINC01391 on tumorigenesis in vivo, we injected HepG2 cells stably transfected with LINC01391 or control vector into the nude mice. The results presented that upregulation of LINC01391 significantly impaired growth compared with control groups, as measured by increased mean tumor volumes and weights (Fig. 3(A) and (B), and (C)). Compared with the control, xenograft tumors contained more of poorly differentiated cells in LINC01391 decrease expressing group and less of poorly differentiated cells in LINC01391 over-expressing group (Fig. 3(D)). The Ki-67 protein is a cellular marker for proliferation; it is strictly associated with cell proliferation [23]. Moreover, IHC staining showed a lower Ki67 proliferation index in the LINC01391 overexpressing tumors than in the control group (Fig. 3(D)).

3.3. LINC01391 interacted with ICAT

To explore the molecular mechanism underlying LINC01391 in HCC, we examined the expression genes involved in HCC associated pathways such as NF-kB, Wnt/β-catenin, and MAPK. However, the qRT-PCR analysis showed that only genes associated with Wnt/β-catenin pathway (Fig. 4(A)) were regulated by LINC01391 instead of NF-kB (p52 and p65) or MAPK pathway(JNK and ERK1/2) (Supplementary Fig. 1(D)).

To further validate the effect of LINC01391 on Wnt/β-catenin, we transfected TOPFLash vector into LINC01391 silenced or overexpressed cells. We observe impaired Wnt/β-catenin activation in LINC01391 overexpressed cells, whereas LINC01391 knockdown cells did the opposite (Fig. 4(B)). Furthermore, subcellular fractionation and qRT-PCR analysis showed that LINC01391 is abundantly expressed in the nucleus (Fig. 4(C)). ICAT can directly bind with β-catenin and impair the interaction between β-catenin and TCF/LEF complex that drives the Wnt/β-catenin pathway. Thus, we hypothesized that LINC01391 might exert a biological function by ICAT. Then, we confirmed the interaction between LINC01391 by RNA pulldown and Western blot (Fig. 4(D)). RNA immunoprecipitation was conducted, and the result showed that LINC01391 was enriched in ICAT samples (Fig. 4(E)). The Mapping assay is a combination of RNA pulldown and Western blot assays which indicated constructed regions of LINC01391 and their interaction with ICAT. Mapping assay in HEPG2 cell lines showed that the second region (701–1400 nt) of LINC01391 was required for its interaction with ICAT (Fig. 4(F)).

ICAT reduced the cell proliferation and apoptosis in vitro and tumor growth

Found that LINC01391 could bind with ICAT. Thus, we further explored the effect of LINC01391 on β-catenin interactomics. Immunoprecipitation was conducted using β-catenin antibodies, and the interaction between β-catenin and ICAT, TCF3, TCF4, and LEF1 was verified by Western blot. LINC01391 silencing cells showed impaired ICAT-β-catenin interaction and enhanced β-catenin-TCF/LEF interaction. Opposite results were observed in LINC01391 overexpressed cells (Fig. 5(A)). Then, we wanted to explore the functional relevance for associations between LINC01391 and ICAT. We did not observe changes in ICAT mRNA levels after changing LINC01391 expression (Fig. 5(B)). However, we detected significant upregulation of ICAT protein in LINC01391 overexpressing HepG2 and SMMC-7721 cells, whereas

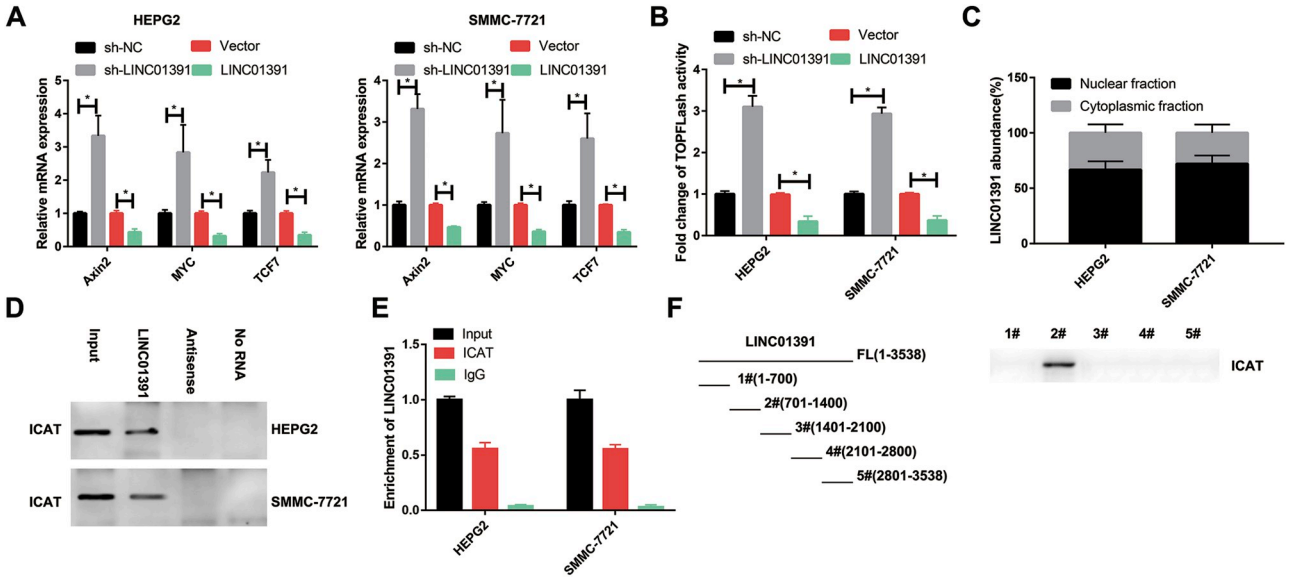

Fig. 4. LINC01391 interacted with ICAT. (A) qRT-PCR was used to measure target gene expression in LINC03191 silenced or overexpressed HepG2 and SMMC-7721 cells. The target genes of Wnt/β-catenin signaling pathway were upregulated in LINC01391 silenced cells but decreased in LINC01391 overexpressed cells. (B) TOPFlash assay of LINC03191 silenced or overexpressed cells. TOPFlash and FOPFlash vectors were transfected into LINC03191 silenced or overexpressed cells, and luciferase intensity was detected 36 h later. (C) qRT-PCR analysis of the subcellular location of LINC01391 in HepG2 and SMMC-7721 cells. U6 (nuclear-retained) and GAPDH (exported to cytoplasm) were used as controls. (D) RNA pull-down assay was performed in HepG2 and SMMC-7721 cells using biotinylated LINC01391 or antisense RNA probe transcribed in vitro and detected by Western blots. (E) RNA immunoprecipitation was performed using ICAT antibody and control IgG, and the enrichments were analyzed using qRT-PCR. (F) RNA pull-down was performed using different LINC01391 deletion probes (top) and the enriched protein extract was detected with Western blots (bottom) in HepG2 cells. Data are shown as the mean ± SEM. *P < 0.05.

LINC01391 knockdown resulted in ICAT downregulation (Fig. 5(C)). Thus, we hypothesized that LINC01391 binded to ICAT and exerted function at the post-transcriptional or translational level. To investigate these hypotheses, we treated LINC01391 overexpressing HepG2 cells with protein synthesis inhibitor cycloheximide (CHX) or proteasome inhibitor MG-132, and found that only MG-132 abolished the changes in ICAT protein levels in LINC01391 overexpressing cells (Fig. 5(D)), thereby suggesting that LINC01391 reduces ICAT protein degradation and enhances its stability.

Finally, we evaluated the function of ICAT in HCC cells using ICAT shRNA. The results demonstrated significantly enhanced cell proliferation and invasion and decreased ratio of apoptotic cells in ICAT silencing cells (Fig. 5(E), (F) and (G)).

Moreover, knockdown of ICAT dramatically enhances the tumor growth in the nude mice (Fig. 5(H), (I) and (J)). The Ki67 proliferation index was lower in the sh-ICAT transfected tumors than in those in the control group (Fig. 5(K)).

4. Discussion

Hepatocellular carcinoma has been the leading cause of cancer death in Asia and Africa. Most patients are already in the advanced stage of hepatocellular carcinoma without effective treatments. Piplartine is a potential anti-tumor drug extracted from pepper. Previous studies have suggested that ROS-ER-MAPKs-CHOP is a key molecular signaling pathway involved in impaired liver cancer cell migration caused by piplartine. In hepatocellular cells, piplartine could activate both p38, JNK, and Erk [15]. We found that the Wnt/β-catenin pathway was inhibited by piplartine in hepatocellular cells by pathway analysis (Supplementary Fig. 2). The Wnt/β-catenin pathway participated in liver tumorigenesis, and liver regeneration and was correlated with poor prognosis and progression [24–26]. β-catenin was released from the complex composed of adenomatous polyposis coli, axin, and glycogen synthase kinase-3β and transferred to the nucleus when activating the Wnt/β-catenin pathway. Intranuclear β-catenin bound to lymphoid-enhancing factor/T-cell factors and activated target genes transcription [27,28]. Therefore, we investigated how piplartine regulated the Wnt/β-catenin pathway.

An increasing number of studies have confirmed that lncRNA could act as regulators of cell proliferation, apoptosis, and metastasis of cancer cells. In bladder cancer, H19 could inhibit H3K27me3 on the DKK1 promoter by interacting with EZH2 [29]. In liver cancer, uc.158 activated the Wnt/β-catenin pathway by competitively binding to miR-193b and promoted the development of liver cancer [30]. Here, we showed that overexpressed LINC01391 inactivated Wnt/β-catenin signaling pathway without affecting the MAPK pathway. This finding indicated that the inhibitory effect of LINC01391 might be mainly based on the Wnt/β-catenin pathway.

ICAT that could directly regulate β-catenin was originally found when screening the mouse embryonic cDNA library. It was encoded by the conserved gene CTNNBIP1 and consisted of only 81 amino acids [31]. ICAT could specifically bind to β-catenin and impaired the activation of TCF, thereby resulting in the inhibition of Wnt/β-catenin pathway. ICAT has elicited increasing attention and was considered a tumor suppressor gene with potential properties for gene therapy or drug development [32,33]. Koyama et al. found that ICAT could inhibit colorectal cancer cells with β-catenin gene mutation proliferation, but this effect did not affect normal cells or wild-type β-catenin gene cells [34,35]. Zhang et al. also obtained similar results in malignant gliomas. ICAT might provide direction for glioma targeted therapy [36]. However, deep research on ICAT indicated that ICAT might also act as an oncogene in other types of cancers. In male genital tumors, overexpressed ICAT was observed in prostate cancer tissues [37]. ICAT might regulate the binding of β-catenin to androgen receptor (AR) and stabilize the AR/β-catenin protein complex, thereby enhancing AR-mediated prostate cancer cell growth [38]. We found that increased expression of LINC01391 could bind to ICAT and promote ICAT expression at the post-transcriptional level. Furthermore, overexpression of LINC01391 could promote the binding of ICAT to β-catenin and inhibit the Wnt/β-catenin pathway.

However, this study has some limitations. When exploring the potential protein binding with LINC01391, we were not able to analyze all

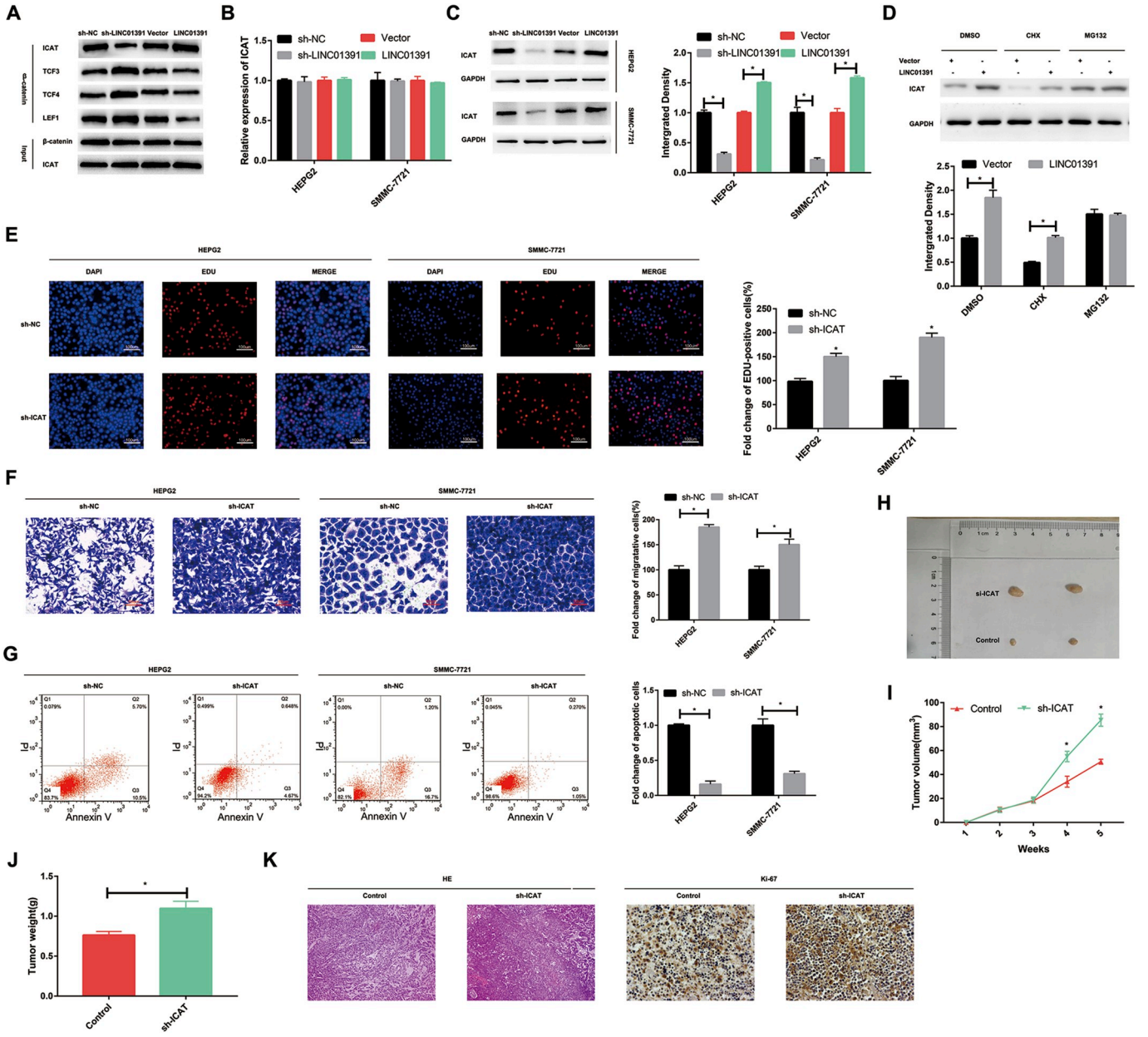

Fig. 5. LINC01391 increases ICAT protein expression by enhancing its stability. (A) β-catenin interaction analyses using co-immunoprecipitation assays with β-catenin antibody. LINC01391 silenced cells and overexpression cells were crushed with RIPA lysis buffer and incubated with β-catenin antibodies. The enrichment samples were analyzed using Western blot with the indicated antibodies. (B) qRT-PCR and (C) Western blot analysis of ICAT expression-based LINC01391 overexpressing or knockdown in HepG2 and SMMC-7721 cells. (D) Stably overexpressing LINC01391 or vector cells were treated with CHX and MG-132, and the ICAT protein expression was measured by Western blots. (E) EDU assays showed that downregulation of ICAT expression promoted the growth of HepG2 and SMMC-7721 cells. (F) Invasion assays of hepatocellular cancer cells with ICAT knockdown. Representative images were shown on the left, and the average number of cells per field was shown on the right. (G) Flow cytometric analysis of the rates of apoptotic hepatocellular cancer cells after knocking down ICAT. (H) The photography of xenograft tumors from mice injected with HepG2 cells transfected with sh-ICAT. (I) Tumor volume curve of mice upon ICAT-knockdown plasmid or vector treatment was analyzed. (J) The weight of mouse tumor was measured. (K) Representative images of immunohistochemical detection of Ki-67 in tumor tissues (Ki-67: a cellular marker for proliferation). Data are shown as mean ± SEM. *P < 0.05.

potential proteins. Based on the role of LINC01391 on the Wnt/β-catenin pathway, we suspected that LINC01391 might bind to ICAT. This combination was validated by RNA-pull down and RIP experiments. However, we were not sure that ICAT was the most important protein for LINC01391 to work. At the same time, we did not investigate the regulation of piplartine on LINC01391. In future studies, if we could analyze the methylation level of the promoter of LINC01391 or the related transcription factors, we might be able to improve the current results. We propose that targeting Wnt/β-catenin signaling would potentially improve the clinical outcomes of cancer patients by overcoming the primary, adaptive, and acquired resistance to immunotherapy [39]. In future work, we can further explore the role of piplartine in immunotherapy of liver cancer and its mechanisms.

In conclusion, we found that piplartine promoted the expression of LINC01391. Furthermore, piplartine and overexpressed LINC01391 inhibited proliferation and invasion and promoted cell apoptosis. Mechanistically, LINC01391 inactivated the Wnt/β-catenin pathway by physical interaction with ICAT and upregulating its expression. Our experimental evidence indicated that piplartine, LINC01391, and ICAT could serve as targets for eradicating HCC.

Declarations

Ethics approval and consent to participate

Not applicable.

Consent for publication

All authors have read and approved the final manuscript and consent to publish.

Availability of data and material

All data generated or analyzed during this study are included in this published article. Further details are available on request.

Competing interests

The authors declare that there is no conflict of interest.

Author contributions

MX and JJ designed the study and experiments. XF, JS, ZZ, MC, JT, CL, FW, DZ, QW, and LZ performed the experiment, collected the data, and conducted the analysis. XF, JS, and ZZ wrote the manuscript draft. MX and JJ revised the manuscript. All of the authors approved the final proof.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.canlet.2019.06.008.

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