Overexpression of IncRNA PIK3CD-AS1 promotes expression of LATS1 by competitive binding with microRNA-566 to inhibit the growth, invasion and metastasis of hepatocellular carcinoma cells

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Abstract

Background: This study is conducted to investigate the effect of IncRNA PIK3CD-AS1 on the growth and metastasis of hepatocellular carcinoma (HCC) and its potential mechanism.

Methods: Hepatocellular carcinoma tissues and adjacent normal tissues together with HCC cells and normal liver cells were obtained for detecting expression of PIK3CD-AS1, microRNA-566 (miR-566) and LATS1. Additionally, a series of experiments were performed to determine cell proliferation, migration, invasion, cell cycle distribution and apoptosis of HCC cells. The xenograft tumor model of HCC was established and the growth rate and weight of xenograft tumor in nude mice were compared. Furthermore, the binding site between PIK3CD-AS1 and miR-566 as well as between miR-566 and LATS1 were verified.

Results: LncRNA PIK3CD-AS1 was downregulated in HCC tissues and cells, and mainly located in cytoplasm. Overexpression of PIK3CD-AS1 inhibited proliferation, colony formation, invasion, migration, epithelial–mesenchymal transition (EMT) and cell cycle progression and promoted apoptosis of HCC cells. Overexpression of PIK3CD-AS1 decreased the growth rate and weight of xenograft tumor in nude mice PIK3CD-AS1 competitively combined with miR-566 to regulate expression of LATS1.

Conclusion: Collectively, our study suggests that the expression of PIK3CD-AS1 was down-regulated in HCC, and overexpression of PIK3CD-AS1 promoted the expression of LATS1 by competitive binding of miR-566 to inhibit the growth, invasion and metastasis of HCC cells.

Keywords: Hepatocellular carcinoma, LncRNA PIK3CD-AS1, LATS1, MicroRNA-566, Growth, invasion, Metastasis
during the past several years [5]. Therefore, it is urgently needed to seek for novel diagnostic biomarkers and therapeutic strategies to improve the prognosis of patients with HCC [6, 7].

Recently, accumulating studies have indicated that lncRNAs could act as competing endogenous RNAs (ceRNAs), which may decrease miRNAs expression and activities, thereby, at the level of post-transcriptional regulation, modulating the derepression of microRNA (miRNA) targets [8–10]. It has been reported that thousands of lncRNAs possess tissue type-, cell type-, disease-specific and developmental stage-expression patterns and localization, implying that individual lncRNA acts as potent natural miRNA sponges in some certain conditions [11]. A study has suggested that the expression of lncRNA PIK3CD-AS1 was significantly decreased in renal cell carcinoma (RCC) [12]. Yet, it is not completely clear whether the specific function of lncRNA PIK3CD-AS1 is implicated in the carcinogenesis of HCC. Numerous studies have demonstrated that lncRNAs and miRNAs could competitively decrease the expression of mRNAs, which may contribute to tumor evolution and progression [8, 13, 14]. The aberrant expression of miRNAs acts an important role in the progression of HCC, and miRNA replacement or inhibition therapies have a great importance on treatments for HCC [15]. A previous study has reported that the expression of miR-566 is related to the prognosis of glioblastoma [16]. However, no reports focused on miR-566 function in HCC till now. Additionally, miRNAs are able to post-transcriptionally modulate the expression of their target genes via their incomplete complementarity with the 3′ non-coding region (3′ UTR) of target mRNAs [17]. Large tumor suppressor gene 1 (LATS1) is able to bind and phosphorylate YAP1 both in vitro and in vivo, influencing its transcription regulation [18, 19]. A previous study has revealed that miR-21 enables to resist radiation therapy by suppressing the expression of LATS1 in ovarian cancer cells [20], while its role in HCC is uncovered. Based on this, we conducted this study to investigate the effect of lncRNA PIK3CD-AS1 on the growth and metastasis of HCC and its potential mechanisms.

Materials and methods

Ethics statement

The study was approved by the ethics committee of The Affiliated Cancer Hospital of Zhengzhou University. The subjects signed the informed consents. Additionally, the animal experiments were also approved by the animal ethics committee of The Affiliated Cancer Hospital of Zhengzhou University. Great efforts have been made to relieve the pain of the animals.

Study subjects

From October 2015 to October 2016, 68 patients with HCC (51 males and 17 females, aged 30 to 70 years, with the median age of 54 years) that were diagnosed and treated in The Affiliated Cancer Hospital of Zhengzhou University were selected into our experiment. The HCC tissues and adjacent normal tissues (at least 3 cm away from the margin of the tumor) were obtained from these patients. All these patients were confirmed to be HCC by pathological examination, and none of them received radiotherapy, chemotherapy, radiofrequency ablation and other clinical adjuvant therapy before operation. Patients with distant metastases or other malignant tumors were excluded.

Cell selection and culture

HCC cell lines (HepG2, Hep3B, Huh7, SMMC-7721, and MHCC-97H) were purchased from Shanghai Institute of Biochemistry and Cell Biology, Chinese Academy of Sciences (Shanghai, China). Human normal liver cell line L-O2 was purchased from Shanghai Oro Biotechnology Co., Ltd. (Shanghai, China). All kinds of cells were cultured in an incubator at 37 °C with 5% CO2 in DMEM medium (Gibco, Grand Island, NY, USA) containing 10% fetal bovine serum (FBS, Gibco, Grand Island, NY, USA). When the cell confluence reached 80–90%, the cells were subcultured once every 2 days. After 2–3 passages, the cells in logarithmic growth phase were obtained. The expression levels of PIK3CD-AS1 and miR-566 in all the cells were detected by reverse transcription quantitative polymerase chain reaction (RT-qPCR). The HepG2 and SMMC-7721 cells with the largest difference from the normal liver cell line L-O2 were selected for subsequent experiment.

Cell transfection, screening and identification

HepG2 and SMMC-7721 cells in logarithmic growth phase were transfected with pcDNA3.1-PIK3CD-AS1 (pcDNA3.1-PIK3CD-AS1 group) or pcDNA3.1 (pcDNA3.1 group) respectively using Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA) according to the instructions provided by the manufacturer. The cells were transferred to another 6-well plate at the ratio of 1:0 and supplemented to the medium containing 500 μg/mL G-418 sulfate or geneticin, and the medium was changed once for 2 days or when the medium was turbid. When a large number of cell death occurred, the concentration of G418 could be reduced by half to maintain the screening pressure. After 10 days of screening, when G418 resistant clones appeared, the drug was stopped and cultured in complete medium, and then the clones grew up gradually. The positive clones were screened by ring
method and limited dilution method. The positive clones were transferred into 6-well plate culture and the positive clones were obtained. The HepG2 and SMMC-7721 stable expression strains of pcDNA3.1-PIK3CD-AS1 and pcDNA3.1 were transfected and screened by this method. The HepG2 and SMMC-7721 stable expression lines of pcDNA3.1-PIK3CD-AS1 and pcDNA3.1 were used to detect the expression of PIK3CD-AS1 in the cells by RT-qPCR. miR-566 mimics or mimics NC were transfected into cells using Lipofectamine 2000 reagent (Invitrogen, Carlsbad, CA, USA) according to manufacturer’s protocol. pcDNA3.1, pcDNA3.1-PIK3CD-AS1, mimics NC and miR-566 mimics were synthesized by Shanghai Sangon Bioengineering, Co., Ltd. (Shanghai, China).

**Nuclear and cytoplasmic separation experiment**

The bioinformatics website [http://lncatlas.crg.eu/](http://lncatlas.crg.eu/) was used to predict the subcellular localization of PIK3CD-AS1. Then the nuclear and cytoplasmic separation experiment was carried out with PARIS™ Kit (Ambion, Austin, Texas, USA). Totally 10⁶ cultured HepG2 and SMMC-7721 cells were collected, washed with PBS twice and placed on ice. The cells were added with 500 μL cell fractionation buffer and incubated on ice for 10 min. After centrifugation for 5 min, supernatant (cytoplasm) and precipitate (nucleus) were separated. The supernatant was preheated with equal volume for 2× Lysis/Binding solution to prevent the degradation of RNA. The anhydrous alcohol of the same volume as the supernatant was added and then added it to the filter column provided by the kit. RNA was collected on filter membrane and centrifuged for 30 s, which became soluble cytoplasm RNA. Nuclear precipitation was performed based on these steps, and the collection was a dissolved nuclear RNA. The collected cytoplasm and nuclear RNA were then reverse transcribed by M-MLV kit. RT-qPCR was used to detect the expression of PIK3CD-AS1 in nucleus and cytoplasm respectively.

**RNA-fluorescence in situ hybridization (FISH)**

The HepG2 and SMMC-7721 cells were placed on the bottom of the 24-well plate at the density of 5 × 10⁵ cells/well. After 24-h culture, the supernatant was removed and 1× PBS was used to clean the cells. After that, cells were fixed by 4% paraformaldehyde, rinsed with PBS containing 0.5% TritonX 100, blocked with prehybridization solution (Guangzhou Ribo Biotechnology Co., Ltd., Guangzhou, China) at 37 °C. PIK3CD-AS1 probe was used at 37 °C for hybridization overnight, and the hybridization solution (Guangzhou Ribo Biotechnology Co., Ltd., Guangzhou, China) was utilized to clean cells at 42 °C. The slice hybridization area was stained with 4',6-diamidino-2-phenylindole 2HCl (a stain) (DAPI) (Guangzhou Ribo Biotechnology Co., Ltd., Guangzhou, China). The slides were fixed under the condition of avoiding light. The specimens were observed under a laser confocal microscope.

**Dual luciferase reporter gene assay**

The binding sites of IncRNA PIK3CD-AS1 and miR-566 were predicted and analyzed by bioinformatics website [https://cm.jefferson.edu/rna22/Precomputed/](https://cm.jefferson.edu/rna22/Precomputed/). The binding site between PIK3CD-AS1 and miR-566 was verified by luciferase activity assay. The synthetic PIK3CD-AS1 3′UTR gene fragment was introduced into pMIR-reporter (Beijing Huayuyang Biotechnology Co., Ltd., Beijing, China) by endonuclease sites Bamh1 and Ecorn. The complementary mutation (MUT) site of seed sequence was designed on PIK3CD-AS1 wild type (WT). The target fragment was inserted into pMIR-reporter plasmid by restriction endonuclease digestion and T4 DNA ligase, followed by co-transfection of sequenced luciferase reporter plasmids WT and MUT with mimics NC and miR-566 mimics into 293T cells (Shanghai Beino Biotechnology Co., Ltd., Shanghai, China). After transfection for 48 h, the cells were collected and lysed, and luciferase assay kit (BioVision, San Francisco, CA, USA) was used to detect luciferase activity by using a GloMax20/20 luminometer (Promega, Madison, Wisconsin, USA). The experiment was repeated three times in each group.

Bioinformatics software [http://www.targetscan.org](http://www.targetscan.org) was used to predict the binding site between miR-566 and LATS1. The sequence of LATS1 3′UTR promoter containing miR-566 binding site was synthesized and the LATS1 3′UTR WT and LATS1 3′UTR-MUT plasmid was constructed. The cells at logarithmic growth were inoculated into a 96-well plate, and lipofectamine 2000 was transfected at about 70% cell confluence LATS1-WT and LATS1-MUT plasmids were mixed with mimics NC and miR-566 mimics plasmids and then co-transfected into 293T cells. After 48 h of transfection, the cells were collected and lysed. Luciferase assay kit was used to detect the luciferase activity. The experiment was repeated three times in each group.

**RNA-pull down**

Biotin-labeled miR-566 WT plasmids and miR-566 MUT plasmids (50 nM each) were transfected into the cells. After 48 h of transfection, the cells were collected and rinsed with PBS, and incubated with a specific cell lysis (Ambion, Austin, Texa Stilla, USA) for 10 min. After that, 50 mL cell lysis was packaged. The residual
pyrolysis was incubated with M-280 streptavidin magnetic beads (Sigma-Aldrich, St. Louis, MO, USA) pre-coated with RNase-free and yeast tRNA (Sigma-Aldrich, St. Louis, MO, USA) at 4 °C for 3 h. Then it was washed twice with cold cracking solution, three times with low salt buffer and once with high salt buffer. Antagonistic miR-566 probe was established as a NC. Total RNA was extracted by Trizol and the expression of PIK3CD-AS1 was detected by RT-qPCR.

**RT-qPCR**

Trizol method (Invitrogen, Carlsbad, CA, USA) was employed to extract total RNA of tissues and cells. The ultra-violet analysis and formaldehyde gel electrophoresis were performed to confirm the high quality of RNA. Then 1 μg RNA was collected, and avian myeloblastosis virus reverse transcriptase was used for reverse transcription to obtain cDNA. The primers of PCR were designed and synthesized by Invitrogen (Carlsbad, CA, USA) (Table 1), with glyceraldehyde-3-phosphate dehydrogenase (GAPDH) as an internal reference. The data were analyzed by $2^{-\Delta\Delta CT}$. The experiment was conducted in triplicate.

**Western blot analysis**

The proteins were transferred onto polyvinylidene fluoride (PVDF) membranes. The membranes were blocked with 5% bovine serum albumin (BSA) for 1 h. Primary antibodies of LATS1, E-cadherin and Vimentin (ab105106, ab15148 and ab16700; 1:1000, Abcam, Cambridge, MA, USA) and primary antibody β-actin (ab227387; 1:5000; Abcam, Cambridge, MA, USA) were added and incubated at 4 °C overnight, followed by three washes (5 min per wash) in Tris-buffered saline with Tween 20 (TBST). Corresponding secondary antibodies (Shanghai Miaoqong Biotechnology Co., Ltd., Shanghai, China) were added and incubated for 1 h. The membranes were washed three times with 5 min for each time. Chemiluminescence reagents were employed to develop images. β-actin was considered as an internal reference. The images of the gels were captured in a Bio-Rad Gel Doc EZ Imager (Bio-Rad, Hercules, CA, USA). The grey values of target protein bands were analyzed by an ImageJ software. The experiment was conducted in triplicate.

**Immunofluorescence staining**

The cells of each group were cultured on glass slides and the inoculation density was 50–60%. After the cells were adhered to the wall, they were rinsed with cold PBS 2 times, fixed in 4% paraformaldehyde at room temperature for 30 min, rinsed with PBS 2 times, and reacted with 0.1% Triton X-100 at room temperature for 10 min. The cells were supplemented with normal goat serum and blocked at room temperature for 1–2 h. E-cadherin and Vimentin antibodies as well as PE-Flag monoclonal antibody (Abcam, Cambridge, MA, USA) were added into the shaking bed at 37 °C for 2 h, and then washed with PBST three times (10 min each time). Subsequently, the cells were stained with DAPI for 3–5 min, rinsed with PBS for 3–5 min, and sealed with mounting medium. The slide was placed under a fluorescent microscope for 30 min at 37 °C.

**Table 1** Primer sequence

| Gene       | Sequence                  |
|------------|---------------------------|
| PIK3CD-AS1 | F: 5′-GGGTGCTCTCTGGAACAGTC-3′ |
|            | R: 5′-GGAGTCTGGTGAGGCTCAAG-3′ |
| miR-566    | F: 5′-UUCGAGACGGGUCGG-3′   |
|            | R: 5′-CTCGCTTGGCAGCACA-3′   |
| U6         | F: 5′-CAACCCTACCCCAACATCTG-3′ |
|            | R: 5′- GGCTGCTGGAGATTGGATGC-3′ |
| LATS1      | F: 5′-ACCACATTGAGAAGGCTCG-3′ |
|            | R: 5′-CTCAGTGAGCTCAGATGC-3′  |
| GAPDH      | F: 5′-ACCACCATGAGAAGGCTCG-3′ |
|            | R: 5′-CTCAGTGAGCTCAGATGC-3′  |

F forward, R reverse, miR-566 microRNA-566, GAPDH glyceraldehyde phosphate dehydrogenase
EdU assay

Cell-light EdU luminescence assay kit (RiboBio, Guangzhou, China) was used to detect the DNA replication ability of cells. After routine treatment of cells in each group, the cells were seeded in a 96-well plate with 1.0 × 10^4 cells/well, with three parallel wells in each group. Afterwards, the cells were incubated with 100 μL EdU (50 μM) for 2 h, rinsed with PBS 2 times, fixed with 4% paraformaldehyde for 20 min, incubated with 2% glycine for 15 min, rinsed with PBS 2 times, permeabilized with 150 μL permuting agent, and rinsed with PBS 3 times. According to the instructions of EdU kit, we continued to treat the cells. Under the fluorescence microscope (Olympus F100), five visual fields were taken randomly. The blue fluorescence represented all the cells, while the red fluorescence was the replicating cells infiltrated by EdU. The percentage of EdU positive cells was calculated.

Colony formation assay

The detached cells were fully dispersed and inoculated with 200 cells into 6-well plates, and the culture plate was gently shaking so that the cells were dispersed evenly and cultured for 2–3 weeks. When the cell colony was visible to the naked eye, the culture was terminated, and the culture solution was abandoned. The cells were rinsed with PBS and fixed with 4% paraformaldehyde for 30 min. After that, the cells were washed with PBS for 3 times, and stained with Giemsa application solution for 60 min. The number of cell colony was counted under a microscope.

Flow cytometry

The detached cells in each group were collected and centrifuged, then the supernatant was discarded. Subsequently, the cells were suspended and washed with PBS to make the cell concentration adjusted to 1 × 10^5 cells/mL, thus the single cell suspension was prepared. The single cell suspension was centrifuged for 5 min at 2000 rpm, and the supernatant was removed. The volume fraction of about 70% ethanol (500 μL) was added to each group, and then fixed for 2 h at 4 °C overnight. The fixative solution was discarded, and 1 mL PBS was supplemented to further eluate fixation solution, centrifuged at 2000 rpm for 3 min, with the supernatant removed. Afterwards, 100 μL RNase A was added to the cells at 37°C for 30 min, and then 400 μL PI was added to the cells and mixed at 4°C for 30 min devoid of light. The red fluorescence at the excitation wavelength of 488 nm was recorded.

After the cells in logarithmic growth phase were detached, the suspension cells were centrifuged to collect the cells, with the supernatant discarded. The cell concentration was adjusted to 10^6 cells/mL, and 200 μL of the cells were washed with 1 mL pre-cooled PBS two times and then centrifuged. The cells were suspended in 100 μL binding buffer and added with 2 μL Annexin-V-FITC (20 μg/mL), gently mixed, and placed on the ice for 15 min. Next, the cells were transferred to a low detection tube, supplemented with 300 μL PBS, and also added with 1 μL PI (50 μg/mL) to each sample before putting on the machine, and the detection was lasted for 30 min. Annexin V was regarded as horizontal axis, and PI as longitudinal axis; left upper quadrant as mechanically injured cells, right upper quadrant as late apoptotic cells or necrotic cells, left lower quadrant as negative normal cells and right lower quadrant as early apoptotic cells.

Transwell assay

The cells in each group were suspended with serum-free DMEM medium containing BSA, and the cell density was adjusted to 1 × 10^5 cells/mL. Subsequently, the cells (200 μL) were incubated in the apical chamber of Transwell (The invasion test was carried out on the polycarbonate membrane of Transwell which was coated with 30 μL Matrigel [1 mg/mL]. Matrigel was reconstituted on the microporous filter membrane to form the basement membrane structure at 37 °C after 60 min). The basolateral chamber was appended with DMEM medium (600 μL) containing 10% FBS. After 24-h incubation, the cells in the apical chamber were erased with cotton swabs at 37 °C with 5% CO2, fixed with 4% paraformaldehyde for 15 min, and stained with crystal violet for 10 min. Under the microscope, five visual fields were taken and the number of cells penetrating the membrane was counted. The experiment was repeated three times.

Establishment of an ectopic transplantation tumor model of HCC in mice

A total of 15 BALB/c nude mice, 4–5 weeks old, purchased from Hunan SLAC Experimental Animal Co., Ltd., (Hunan, China), were raised under SPF conditions with sufficient food and water. After 1 week, the state of the animals was observed every day. Fifteen nude mice were divided into three groups: blank group, pcDNA3.1 group and pcDNA3.1-PIK3CD-AS1 group, five rats in each group. SMMC-7721 cells with better growth status and stable overexpression of pcDNA3.1-PIK3CD-AS1 and pcDNA3.1 empty plasmid was obtained. After trypsinization, the cells were collected, and rinsed with normal saline. The cells were made into cell suspension, and the cell suspension was aspirated under sterile conditions using a medical sterile syringe (1 mL). The roots of the hind legs of mice in each group were inoculated with 100 μL single cell suspension (2 × 10^5 cells) at a concentration of 2 × 10^5 cells/mL, and the cell suspension
was mixed before injection. The nude mice were kept under aseptic conditions, which were sacrificed when the transplanted tumor grew to a size of 1.0 to 1.5 cm. The transplanted tumor was removed, and the volume of the transplanted tumor was measured and weighed.

**Statistical analysis**
The data were analyzed by SPSS21.0 software (IBM-SPSS, Inc, Chicago, IL, USA). The data were normally distributed by Kolmogorov–Smirnov test. The results were expressed as mean ± standard deviation. The comparison between the two groups was performed by the t test, and the comparison among multiple groups were analyzed by one-way analysis of variance (ANOVA). After ANOVA analysis, the Fisher’s least significant difference t test (LSD-t) was utilized for pairwise comparison. All tests were bilateral, and the significant difference was defined as *P* < 0.05.

**Results**
**Expression of LncRNA PIK3CD-AS1 is poor in HCC tissues and cells, and mainly located in cytoplasm**
The expression of PIK3CD-AS1 in HCC tissues and adjacent normal tissues was detected by RT-qPCR. The results showed that the expression of PIK3CD-AS1 in HCC tissues was significantly lower than that in corresponding adjacent normal tissues (Fig. 1a; *P* < 0.01).

RT-qPCR results also suggested that the expression of PIK3CD-AS1 in HCC cells (HepG2, Hep3B, Huh7, SMMC-7721 and MHCC-97H) was significantly lower than that in human normal liver cell (L-O2) (all *P* < 0.05). Meanwhile, the expression of PIK3CD-AS1 was the lowest in HepG2 and SMMC-7721 cells, which was the most different from that in L-O2 cells. Therefore, HepG2 and SMMC-7721 cells were selected for subsequent experiment (Fig. 1b).

The subcellular localization of PIK3CD-AS1 was predicted by bioinformatics website (http://lncatlas.crg.eu)/. The results showed that the expression of PIK3CD-AS1 in SMMC-7721 cells was mainly located in the nucleus, but there was no study concentrated on HCC and other tumor cells (Fig. 1c).

The subcellular localization of PIK3CD-AS1 in HepG2 and SMMC-7721 cells was analyzed by nuclear and cytoplasmic separation experiments. The results demonstrated that PIK3CD-AS1 was expressed in the nucleus and cytoplasm of HepG2 and SMMC-7721 cells. However, the expression level in cytoplasm was relatively high, and the expression level in nucleus was comparatively low (*P* < 0.01), suggesting that PIK3CD-AS1 played a major role in the cytoplasm of HCC (Fig. 1d).

Further, the subcellular localization of PIK3CD-AS1 in HepG2 and SMMC-7721 cells was detected by FISH. The results revealed that PIK3CD-AS1 was expressed...
in the nucleus and cytoplasm of HepG2 and SMMC-7721 cells, and the cytoplasmic expression was more than the nucleus (Fig. 1e).

The expression of PIK3CD-AS1 in HepG2 and SMMC-7721 cells in the pcDNA3.1-PIK3CD-AS1 group was significantly higher than that in the blank group (all \( P < 0.01 \)), 81.7% and 78.8% higher than that in the blank group, respectively. No significant difference was found in expression of PIK3CD-AS1 between the blank and the pcDNA3.1 group (\( P > 0.05 \); Fig. 1f). The results implied that HepG2 and SMMC-7721 cells stably expressing PIK3CD-AS1 were successfully constructed with high transfection efficiency.

**Overexpression of PIK3CD-AS1 inhibits the proliferation and colony formation of HCC cells**

The results of CCK-8 assay showed that the proliferation rate of HepG2 and SMMC-7721 cells in the pcDNA3.1 group was not significantly different from that in the blank group (all \( P > 0.05 \)), but the proliferation rate of cells in the pcDNA3.1-PIK3CD-AS1 group was significantly decreased relative to the blank group (all \( P < 0.05 \); Fig. 2a).

EdU assay was used to detect the replication activity of DNA. The results suggested that the replication activity of DNA in the blank group was the same as that in the pcDNA3.1 group, but the replication activity of DNA decreased significantly after transfection of pcDNA3.1-PIK3CD-AS1 (\( P < 0.05 \); Fig. 2b).

The colony forming ability of HepG2 and SMMC-7721 cells in each group was detected by colony forming assay. The results revealed that there was no significant difference in the number of colony formation between the blank group and the pcDNA3.1 group (\( P > 0.05 \)), but the number of colony formation of cells in the pcDNA3.1-PIK3CD-AS1 group was significantly lower than that in the blank group (all \( P < 0.05 \); Fig. 2c). The results showed that overexpression of PIK3CD-AS1 could inhibit the ability of colony formation of HCC cells.

**Overexpression of PIK3CD-AS1 inhibits the cell cycle progression and promotes apoptosis in HCC cells**

The cell cycle and apoptosis of HepG2 and SMMC-7721 cells were determined by flow cytometry. The proportion of cells in the blank group and the pcDNA3.1 group was not statistically different (\( P > 0.05 \)). Compared with the blank group, more cells arrested at the G0/G1 phase, while fewer cells arrested at G2/M phase in the pcDNA3.1-PIK3CD-AS1 group (all \( P < 0.05 \); Fig. 3a). The apoptosis rate of cells in the pcDNA3.1-PIK3CD-AS1 group was significantly higher than that in the blank group (all \( P < 0.05 \); Fig. 3b).
group was significantly higher than that in the blank group and the pcDNA3.1 group (all \( P < 0.05 \); Fig. 3b). These results suggest that overexpression of PIK3CD-AS1 can inhibit the cell cycle progression and promote apoptosis of HCC cells.

**Overexpression of PIK3CD-AS1 inhibits epithelial–mesenchymal transition (EMT) in HCC cells**

The changes of cell morphology in each group were observed under an inverted microscope. In HepG2 and SMMC-7721 cells, transfection of pcDNA3.1 had no effect on the epithelial mesenchymal morphology, while transfection of pcDNA3.1-PIK3CD-AS1 promoted the transformation of mesenchymal morphology from fusiform and multi-protruded mesenchymal cells to soft circular epithelial-like morphology (Fig. 4a).

RT-qPCR findings showed that there was no significant difference in the expression of ZEB1 and Snail mRNA in HepG2 and SMMC-7721 cells between the blank group and the pcDNA3.1 group (all \( P > 0.05 \)). Additionally, the expression of ZEB1 and Snail mRNA in cells of the pcDNA3.1-PIK3CD-AS1 group was significantly lower than that in the blank group (all \( P < 0.05 \); Fig. 4b).

The results of immunofluorescence staining suggested that there was no significant difference in the expression of E-cadherin and Vimentin between the blank group and the pcDNA3.1 group (all \( P > 0.05 \)). The expression of E-cadherin was significantly higher while the expression of Vimentin was lower in cells of the pcDNA3.1-PIK3CD-AS1 group than that in the blank group (Fig. 4c). These results suggest that overexpression of PIK3CD-AS1 can inhibit the EMT of HCC cells.

The results of western blot analysis indicated that the expression of E-cadherin was increased in HepG2 and SMMC-7721 cells with stable overexpression of PIK3CD-AS1, while the expression of Vimentin was decreased in contrast to the blank group (all \( P < 0.05 \); Fig. 4d).

**Overexpression of PIK3CD-AS1 inhibits invasion and migration of HCC cells**

Transwell assay was used to detect the invasion and migration ability of HepG2 and SMMC-7721 cells. The number of invasive cells in the blank group and the pcDNA3.1 group was similar (\( P > 0.05 \)). The number of cell invasion in the pcDNA3.1-PIK3CD-AS1 group was significantly lower than that in the blank group and the pcDNA3.1 group (all \( P < 0.05 \); Fig. 5a). Similarly, the number of cell migration in the pcDNA3.1-PIK3CD-AS1 group was significantly lower than that in the blank group and pcDNA3.1 group (all \( P < 0.05 \); Fig. 5b). These results suggest that overexpression of PIK3CD-AS1 can reduce the invasion and migration of HCC cells.
Fig. 4 Effect of overexpression of PIK3CD-AS1 on EMT of hepatocellular carcinoma cells. a Observation of cell morphology by an inverted microscope (×200); b detection of ZEB1 and Snail mRNA expression in cells by RT-qPCR; c detection of E-cadherin and Vimentin protein expression in cells by immunofluorescence staining (×400); d detection of E-cadherin and Vimentin protein expression by western blot analysis; the experiment was repeated three times; the data was analyzed by the t test or one-way ANOVA, and after ANOVA, the pairwise comparison was analyzed by the LSD-t test; *P < 0.05 vs the blank group.
Molecular mechanism of PIK3CD-AS1 regulating the function of HCC cells

RT-qPCR was used to detect the expression of miR-566 in HCC tissues and adjacent normal tissues. The results indicated that the expression of miR-566 in HCC tissues was significantly higher than that in corresponding adjacent normal tissues \( (P<0.05); \) Fig. 6a). The expression of miR-566 in HCC cells was significantly higher than that in normal liver cells (all \( P<0.05); \) Fig. 6b). It is suggested that PIK3CD-AS1 and miR-566 may have a negative correlation in HCC.

To further detect the expression of miR-566 in HepG2 and SMMC-7721 cells with stable overexpression of PIK3CD-AS1, we found that the expression of miR-566 decreased significantly in HepG2 and SMMC-7721 cells with stable overexpression of PIK3CD-AS1 (all \( P<0.05); \) Fig. 6c). It is confirmed that the expression of miR-566 is negatively regulated by PIK3CD-AS1 in HCC.

The specific binding region between PIK3CD-AS1 and miR-566 sequences was predicted by online analysis software (Fig. 6d). The results of dual luciferase reporter gene assay revealed that luciferase activity in WT-PIK3CD-AS1 + miR-566 mimics group was significantly lower than that in the mimics NC group \( (P<0.05), \) and luciferase activity in MUT-PIK3CD-AS1 + miR-566 mimics group did not change significantly \( (P>0.05), \) indicating that there was a binding relationship between PIK3CD-AS1 and miR-566 (Fig. 6e).

The results of RNA-pull down indicated that the PIK3CD-AS1 expression in the Bio-miR-566-WT group was significantly higher than that in the Bio-probe NC group in HepG2 and SMMC-7721 cells \( (P<0.05). \) There was no significant difference in PIK3CD-AS1 expression in the Bio-miR-566-MUT group and the Bio-probe NC group \( (P>0.05). \) The results showed that Bio-miR-566-WT could promote the enrichment of PIK3CD-AS1 around it, but Bio-miR-566-MUT could not. It was proved that PIK3CD-AS1 combined with miR-566, which could reduce the degree of miR-566 dissociation (Fig. 6f).

PIK3CD-AS1 competitively combined with miR-566 to regulate expression of LAST1

The results of RT-qPCR and western blot analysis were used to detect the mRNA and protein expression of LATS1 in HCC tissues and cells. The results suggested that the mRNA and protein expression of LATS1 in HCC tissues was significantly lower than that in adjacent normal tissues (both \( P<0.05); \) Fig. 7a). Compared with normal liver cells, the mRNA and protein expression of LATS1 significantly decreased in HCC cells (all \( P<0.05); \) Fig. 7b). Combined with the expression number of invasion cells

\[
\begin{array}{c|c|c}
\text{Condition} & \text{HepG2} & \text{SMMC-7721} \\
\hline
\text{Blank} & 60 & 50 \\
\text{pcDNA3.1} & 40 & 40 \\
\text{pcDNA3.1-PIK3CD-AS1} & 20 & 20 \\
\end{array}
\]

\[\text{Number of migration cells:}\]

\[
\begin{array}{c|c|c}
\text{Condition} & \text{HepG2} & \text{SMMC-7721} \\
\hline
\text{Blank} & 150 & 150 \\
\text{pcDNA3.1} & 100 & 100 \\
\text{pcDNA3.1-PIK3CD-AS1} & 50 & 50 \\
\end{array}
\]

Fig. 5 Effect of overexpression of PIK3CD-AS1 on invasion and migration of hepatocellular carcinoma cells. a Transwell assay for cell invasion in HCC cells \((x \times 400);\) b transwell assay for cell migration in HCC cells \((x \times 400);\) the experiment was repeated three times; the data was analyzed by the t test or one-way ANOVA, and after ANOVA, the pairwise comparison was analyzed by the LSD-t test; \( *P<0.05 \) vs the blank group.
of PIK3CD-AS1, the positive correlation between PIK3CD-AS1 and LATS1 may exist in HCC.

Bioinformatics software (http://www.targetscan.org) predicted a targeted relationship between miR-566 and LATS1 (Fig. 7c). The results of luciferase activity assay suggested that the relative luciferase activity of the cells significantly decreased after LATS1-WT and miR-566 mimics were co-transfected into 293T cells (P < 0.05), while cells that co-transfected with LATS1-MUT and miR-566 mimics did not affect the relative luciferase activity of the cells (P > 0.05) (Fig. 7d). This indicates that LATS1 is a direct target gene of miR-566.

To verify the relationship between PIK3CD-AS1, miR-566 and LATS1, we detected the mRNA and protein expression of LATS1 in HepG2 and SMMC-7721 cells that expressed with stable PIK3CD-AS1. The results indicated that the LATS1 mRNA and protein expression significantly increased in HepG2 and SMMC-7721 cells transfected with miR-566 mimics were significantly decreased (all P < 0.05). At the same time, we performed rescue experiments using miR-566 mimics in cells with PIK3CD overexpression. The results indicated that the pcDNA3.1-PIK3CD-AS1 + miR-566 mimics group and the miR-566 groups exhibited decreased mRNA and protein expression of LATS1 relative to the pcDNA3.1-PIK3CD-AS1 group. Therefore, we found that PIK3CD-AS1 positively regulated the expression of LATS1, and miR-566 inhibited the expression of LATS1 in HCC. Based on this, we confirmed that PIK3CD-AS1 could inhibit the expression of LATS1 by competitive binding to miR-566 in HCC, thereby affecting the occurrence and development of HCC.

Overexpression of PIK3CD-AS1 inhibits the growth of transplanted tumors in HCC

To further observe the effect of PIK3CD-AS1 on the growth of transplanted tumors, SMMC-7721 cells
Fig. 7 Expression of LATS1 in HCC tissues and cells and verification of the targeting relationship between miR-566 and LATS1. a Detection of mRNA and protein expression of LATS1 in HCC and adjacent normal tissues by RT-qPCR and western blot analysis, n = 68; b RT-qPCR and western blot analysis were used to detect the expression of LATS1 in HCC cells and normal liver cells; c prediction of targeting relationship between miR-566 and LATS1 by bioinformatics software; d luciferase activity assay to verify the targeting relationship between miR-566 and LATS1; e RT-qPCR and western blot analysis were used to detect the expression of LATS1 in HCC cells of each group; the experiment was repeated three times; the data was analyzed by the t test or one-way ANOVA, and after ANOVA, the pairwise comparison was analyzed by the LSD-t test; *P < 0.05 vs L-O2 cells or the blank group.
stably overexpressing PIK3CD-AS1 and pcDNA3.1 were injected into the skin of nude mice, and the volume of transplanted tumors was monitored every 5 days. The results suggested that the volume of transplanted tumors in the pcDNA3.1-PIK3CD-AS1 group was significantly smaller than that in the blank and the pcDNA3.1 groups (P < 0.05), but there was no significant difference in the volume of transplanted tumors between the blank and the pcDNA3.1 groups (P > 0.05) (Fig. 8a). After 25 days, the nude mice were sacrificed, the transplanted tumor was removed and weighed. The results suggested that the weight of transplanted tumors in the pcDNA3.1-PIK3CD-AS1 group was significantly smaller than that in the blank and the pcDNA3.1 groups (P < 0.05), but there was no significant difference in the weight of transplanted tumors between the blank and the pcDNA3.1 groups (P > 0.05) (Fig. 8b, c). The results suggest that overexpression of PIK3CD-AS1 inhibits the growth of xenografts in HCC.

Discussion

In recent years, IncRNAs have been described as abundant regulators of tumor physiology in HCC with their varying functions [21, 22]. On the one hand, miRNAs play vital roles in the control of cell differentiation, proliferation, invasion, as well as metabolism [23]. On the other hand, it has been indicated that IncRNAs perform both physiological and pathological biological functions in cells [24]. IncRNAs are reported to perform their functions via various mechanisms, such as epigenetic silencing, IncRNA–protein interaction, IncRNA–miRNA interaction, as well as IncRNA–mRNA interaction [25]. Therefore, we performed this study to explore the potential role of one of the IncRNA, PIK3CD-AS1, in the progression of HCC cells through modulating the expression of miR-566. The obtained results demonstrated that the expression of PIK3CD-AS1 was down-regulated in HCC, and overexpression of PIK3CD-AS1 inhibited the growth, invasion and metastasis of HCC cells through promoting the expression of LATS1 by competitive binding to miR-566.

One of the most important findings in our study suggested that increased expression of PIK3CD-AS1 and LATS1 and decreased expression of miR-566 were found in HCC. Evidence has suggested that PIK3CD-AS1 may be a tumor suppressor gene, showing a poor expression in RCC. Besides, PIK3CD-AS1 can inhibit the proliferation and promote apoptosis in RCC cells [12]. Similar to our study, multiple studies have revealed that some IncRNAs, such as IncRNA HULC, RERT and HOTTIP/ HOXA13, are downregulated in HCC, which were closely associated with tumorigenesis, metastasis, diagnosis, prognosis, and drug resistance [22, 26, 27], implying that these studies opened up a new avenue for figuring out the molecular mechanisms for occurrence and development of HCC. Zhang et al. [16] proposed that miR-566 was upregulated in glioma cell lines, and the suppression of miR-566 inhibited the proliferative and invasive abilities of glioma cells through the EGFR/Akt pathway. Meanwhile, the expression levels of miR-566 were significantly downregulated in the lenti-AS-566-infected cells in contrast to the untreated and lenti-negative control (NC)-infected cells [28]. In consistent with our results,
a growing number of studies have documented that the inhibition of LATS1 can result in the occurrence of colorectal cancer, gastric cancer, and renal cell carcinoma [29–31]. Additionally, our study also demonstrated that overexpression of PIK3CD-AS1 inhibited proliferation, colony formation, invasion, migration, EMT and cell cycle progression and promoted apoptosis of HCC cells. Additionally, PIK3CD-AS1 competitively combined with miR-566 to regulate expression of LAST1, and miR-566 inhibited the expression of LATS1 in HCC. The abnormal expression of lncRNAs via the interactions with miRNAs or mRNAs is implicated in the modulation of tumor progression together with tumor biological behaviors in HCC [27, 32, 33]. It is reported that the cancer specific lncRNAs may also have a great influence on the invasion and metastasis of HCC [34]. For example, the expression of lncRNA HULC plays a role in an auto-regulatory loop, and it’s inhibitory to expression and activity of miR-372 is able to upregulate HULC expression in HCC [35]. In addition, multiple lncRNAs, such as lncRNA HOTAIR, MALAT1, and LET, are involved in regulating HCC invasion and metastasis [36–38]. Meanwhile, several lncRNAs, such as HULC, loc285194, and GASS, have also been identified as miRNA targets in several cancers [35, 39, 40]. Evidence has suggested that lncRNAs function as the ceRNAs and sequester the common miRNAs, thereby preventing the miRNAs by binding to their ancestral gene [41]. A previous study has demonstrated miR-566 is able to bind to the complimentary binding sites in the 3’-UTR of Von Hippel–Lindau (VHL) [16]. It has also been proposed that miR-21 enables to resist radiation therapy through inhibiting the expression of LATS1 in ovarian cancer cells [20]. Additionally, miR-21 is able to regulate LATS1 expression in renal cancer, thereby contributing to inhibited proliferation, invasion, as well as cancer stem cell phenotype [42]. Similar to our study, a study has indicated that lncRNA-ATB, as an essential regulator of the invasion–metastasis cascade, is capable of inducing cell invasion by acting as a ceRNA of ZEB and facilitating the colonization of disseminated HCC cells by binding to interleukin-11 mRNA [43]. Also, a recent study elucidated that HULC functions as a ceRNA to induce EMT by sponging miR-200a and then upregulating ZEB1 expression [44]. In accordance with the results in cell experiments, the in vivo experiments indicated that overexpression of PIK3CD-AS1 inhibits the growth of transplanted tumors in HCC.

Conclusion

In conclusion, our study provides evidence that overexpression of PIK3CD-AS1 inhibited the growth, invasion and metastasis of HCC cells through upregulating LATS1 by competitive binding with miR-566. However, more efforts are required to better elucidate the function mechanisms of specific lncRNAs in the progression of HCC, which could undoubtedly enhance our understanding in the development of HCC and further facilitate the development of lncRNA-directed diagnosis and therapy for HCC.

Abbreviations

HCC: hepatocellular carcinoma; EMT: epithelial–mesenchymal transition; LATS1: large tumor suppressor gene 1; AMV: avian myeloblastosis virus; GAPDH: glyceraldehyde-3-phosphate dehydrogenase; BCA: bicinchoninic acid; PVDF: polyvinylidene fluoride; BSA: bovine serum albumin; TBST: Tris-buffered saline with Tween-20; CCK-8: cell counting kit-8.

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Competing interests

The authors declare that they have no competing interests.

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