

Original Article

Downregulation of LINC00673 promotes tumor proliferation via repression of HNF1A in human pancreatic ductal adenocarcinoma

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Abstract: Background: The significance of long intergenic non-coding RNA 673 (LINC00673) in the potential prognosis of PDAC is unclear. In this study, we aimed to evaluate the expression of LINC00673 and its clinical association with PDAC. Then, we explored the potential value of LINC00673 in prognosis of PDAC. Methods: The expression levels of LINC00673 were validated using a quantitative real-time polymerase chain reaction (qRT-PCR) in 51 cases of PDAC and 23 cases of adjacent pancreatic tissues. Then, the potential associations between the expression levels and the clinicopathological characteristics of PDAC tissues were explored. Moreover, the value of LINC00673 was confirmed by constructing a survival curve and a receiver operating characteristic (ROC) curve. Finally, cell proliferation was validated using CCK-8 and EDU. Results: LINC00673 was significantly downregulated in PDAC tissues compared with adjacent pancreatic tissues, and its level was significantly downregulated in three PDAC cell lines in comparison with HPDE cell line. Low expression of LINC00673 was correlated with larger diameters, higher incidences of lymphatic metastases and poor differentiation. Moreover, the area under ROC curves of LINC00673 signature was 0.714. In addition, patients with low LINC00673 expression had a poor overall survival compared with the high LINC00673 group by Kaplan-Meier survival analysis. Finally, knockdown of LINC00673 promoted cell proliferation of PDAC via repression of HNF1 homeobox A (HNF1A) in vitro. Conclusion: We first identified that PDAC patients with low LINC00673 expression had a poor overall survival. These results reveal that LINC00673 may serve as a potential tumor suppressor for prognosis of PDAC patients.

Keywords: Tumor suppressor, long non-coding RNA, LINC00673, PDAC

Introduction

In developed countries, pancreatic cancer (PC) has an abnormally high mortality rate, and it is the fifth most common cause of cancer mortality [1]. In the USA, PC accounts for the fourth largest number of cancer-related deaths, indicates a 5-year survival rate of less than 3%, and has the worst consequence of all cancers [2]. Despite technological advancement in the diagnosis of and therapy for PC, only approximately 4% of those with PC survive 5 years after a PC diagnosis [3]. At present, radical surgical resection is the only prospect of cure for PC, but only about 10-20% of PC patients are candidates for surgery [4]. PC is prone to rapidly invade ambient organs and demonstrates early metastatic transmission [5].

In tumorigenesis, long non-coding RNAs (lncRNAs) are a multitudinous class of appropriately conserved and non-protein-coding RNAs with essential roles [6]. A number of studies have posited that the disorders of lncRNAs are directly linked to human illnesses, including various types of cancers (e.g., bladder, breast, colorectal and lung cancer) [7].

LINC00673 could play crucial role in non-small cell lung cancer (NSCLC) progression and might be a potential therapeutic target for patients with NSCLC [8]. Analysis of approximately 7000 recently genotyped individuals within the PC Case-Control Consortium authenticates a novel locus at 17q25.1 (rs7214041 of LINC00673; the value of OR was 1.38; the 95% CI was 1.26-1.51; the value of *P* was 1.95×10^{-10}), markedly

related with a PC hazard using genome-wide association study [9]. Moreover, HaploReg v2 also indicates that rs7214041 alters regulatory motifs for HNF1A and a recent study of the pancreatic cancer transcriptome suggests that HNF1A may act as a tumor suppressor in pancreatic cancers [9-11]. Furthermore, variants in HNF1A is identified as risk factors for pancreatic cancer in pathway-based and candidate SNP-based analyses of the PanScan data [12, 13], and HNF1A knockdown activated Akt and its downstream target, the mammalian target of rapamycin (mTOR) in pancreatic cancer cells [14]. The PI3K/Akt/mTOR signalling axis plays a critical role in regulating cell proliferation, apoptosis, angiogenesis and metastasis, which is central to the development and maintenance of cancer cells. Aberrant PI3K/Akt signaling is common in pancreatic cancer [15, 16]. It is known that phosphorylation of Akt at Ser 473 is associated with resistance to apoptosis by controlling subcellular localization of pro-apoptotic proteins and mTOR, a serine-threonine kinase, is regulated by phosphorylation on Ser2448 in response to PI3K/Akt oncogenic signaling [17]. In this study, the relationships between the expression of LINC00673 and the clinicopathological parameters of PDAC were explored using the Statistical Product and Service Solutions (SPSS) 13.0 software package. In addition, the value of LINC00673 in PDAC was validated using receiver operating characteristic (ROC) curve and survival curve. Finally, knockdown of LINC00673 was used to validate the effects of LINC00673 to cell proliferation of PDAC via repression of HNF1A.

Materials and methods

Collection of clinical samples and statement of ethics

The clinical data were obtained from 74 patients who underwent PDAC radical resection surgeries from February 2012 to December 2015 at the Chongqing Southwest Hospital. The specimens were soaked in a container with liquid nitrogen immediately after surgery and then placed in an icebox at -80°C until used. No patient had a history of receiving either radiotherapy or chemotherapy before the surgery. The tissues from the patients were observed by pathologists to diagnose PDAC; no other co-occurring cancers were diagnosed. To obtain the adjacent nontumorous tissues, samples were resected and evaluated by pathologists.

This research was authorised by the Human Research Committee of Ethics from Chongqing Southwest Hospital. Every patient signed an informed consent. TNM classification was evaluated by the standard from the Union for International Cancer Control (6th ed., 2002).

Cell culture

The cell lines of PDAC (CFPAC-1, Hs-766T and CaPan-1) and the cell line of HPDE were obtained from ATCC (Manassas, VA, USA). Every cell line was kept in 10% foetal bovine serum (GIBCO, Carlsbad, CA, USA), mixed in RPMI 1640 medium (GIBCO, Carlsbad, CA, USA), supplemented with 50 U/ml streptomycin and 50 U/ml penicillin and then cultured in a humidified 5% CO₂ atmosphere and an incubator at 37°C.

RNA extraction

RNA was collected from the fresh cultured cells and frozen cancerous/adjacent specimen's tissues using the reagent of TRIzol Company (Invitrogen, CA, USA) following the manufacturer's directions. Then, 20-50 µl water treated by diethylpyrocarbonate was used to resuspend and dissolve the RNA. To prove the RNA integrity of the tissues, gel electrophoresis of ethidium bromide was performed and then photographed with a camera from Vilber Lourmat (Marne-la-Vallée, France). Finally, the Smart-Spec Plus spectrophotometer (Bio-Rad, Hercules, CA) was used to measure the purity and concentration of the total RNA.

Reverse transcription

Following the manufacturer's directions, a gDNA Eraser of PrimeScrip RT reagent Kit (TaKaRa, Dalian, China) was used to turn the total RNA into cDNA.

Quantitative real-time PCR

Real-time PCR was executed on a CFX96 Real-Time System (Bio-Rad, CA, USA) using the SYBR Premix Ex Taq™ II (TaKaRa, Dalian, China) according to the manufacturer's instructions. Briefly, 12.5 µl Taq II of SYBR Premix Ex, 2 µl cDNA, 1 µl pcr reverse primer, 1 µl pcr forward primer and 8.5 µl DEPC-treated water were added in the system under condition of 95°C for 30 s, then 40 cycles at 95°C for 5 s, and 60°C for 30 s. Glyceraldehyde-3-phosphate

dehydrogenase (GAPDH) acted as a reference for LINC00673. Each sample was repeated in triplicate. Primers were designed on the web (<http://www.ncbi.nlm.nih.gov/tools/primer-blast/>), and they were compounded by a company (Invitrogen, CA, USA). The primer of LINC00673 forward was 5'TCAGAAGACCCACGACCTCT3', LINC00673 reverse was 5'AA-TACCTCCAGCTTGGCGAC3'; HNF1A forward was 5'GCCCTGTACACCTGGTACG3' and HNF1A reverse was 5'CGCCCCTTCTTGGTTGGTAG3'; GAPDH forward was 5'GGAGCGAGATCCCTCCAAAAT3' and GAPDH reverse was 5'GGCTGTTGCATACCTTCTCATGG3'. The specific amplification was analyzed by melt curve of qRT-PCR.

The siRNA of LINC00673

The siRNA sequence of human LINC00673 was provided by GenePharma (Shanghai, China). The sequence of the siRNA for LINC00673 was sense: 5'-GGCGAAUAGUUGGCUUUCUTT-3' and reverse 5'-AGAAAGCCAACUAUUCGCCTT-3'. The sequence of the negative control was sense: 5'-UUCUCCGAACGUGUCACGUTT-3' and reverse 5'-ACGUGACAGUUCGGAGAATT-3'.

Cell proliferation assays

Cell Counting Kit-8 (CKK-8) (Biosharp, Hefei, China) and 5-Ethynyl-2'-deoxyuridine (EDU) kit were used to assess cell proliferation. Transfected cells were plated in each well of a 96-well plate and assessed according to the manufacturer's instructions.

Western blotting

Total protein was extracted from cultured cells using RIPA buffer containing phenylmethane-sulfonylfluoride (PMSF) (Beyotime, Nantong, China). Protein samples were loaded equally in each lane, then resolved using SDS-PAGE (Beyotime, Nantong, China), and transferred onto a nitrocellulose membrane. The membranes were blocked with 5% BSA for 1 h at room temperature and incubated at 4°C overnight with primary antibodies. The primary antibodies used include: HNF1A from BD Biosciences (San Jose, CA) at 1:400 dilution; phospho-AKT (Ser473) at 1:200; and phospho-mTOR (Ser2448) at 1:300 dilution from Cell Signaling (Danvers, MA). Beta-actin at 1:3000 dilutions was used as the loading control. After incubation with appropriate secondary antibodies

conjugated to horseradish peroxidase, the membranes were exposed to ECL Western Blotting detection reagent. Membranes were stripped for 30 minutes at 55°C in a buffer containing 2% SDS, 62.5 mM Tris (pH 6.7), and 100 mM 2-mercaptoethanol for staining of multiple proteins. Staining intensities were quantified by densitometric analysis.

Statistical analysis

To evaluate the association between LINC00673 expression levels and clinicopathological parameters, SPSS 13.0 software package (IBM, NY) was used by the chi-square test. Then, Mann-Whitney U test was used to explore the differences of LINC00673 expression between PDAC tissues and adjacent pancreatic tissues. To evaluate the diagnostic value of LINC00673, the curve of ROC was generated for distinguishing between PDAC tissues and adjacent pancreatic tissues. Pearson correlation analyses were used to investigate the correlation between LINC00673 and HNF1A mRNA expression. Paired-Samples T Test was used to explore the differences of LINC00673 level of 39 paired samples. Statistical significance was set at $P < 0.05$. All of the pictures were plotted using SPSS 13.0 (IBM, NY) and GraphPad Prism 5 (GraphPad, CA, USA).

Results

LINC00673 expression was significantly decreased in PDAC tissues and cell lines

We found that LINC00673 was significantly downregulated in PDAC tissues compared with corresponding normal tissues from 39 paired samples using Expression profiling array of GEO profiles (**Figure 1C**) (http://www.ncbi.nlm.nih.gov/geo/tools/profileGraph.cgi?ID=GDS4103:230812_at). The chromosomal location of its gene is 17q24.3. Its transcript length is 2275 bp. It is also called "Human Islet Long Non Coding RNA 75". However, its associations with the clinical characteristics of PDAC are unclear. To confirm the LINC00673 expression levels in PDAC, we detected the total RNA extracted from 51 cases of pancreatic tumour tissues and 23 cases of adjacent pancreatic tissues by qRT-PCR. The results indicated that the standard of LINC00673 was significantly downregulated in PDAC tissues compared with adjacent pancreatic tissues ($P = 0.003$, **Figure 1A**). In

LINC00673 expression in PDAC

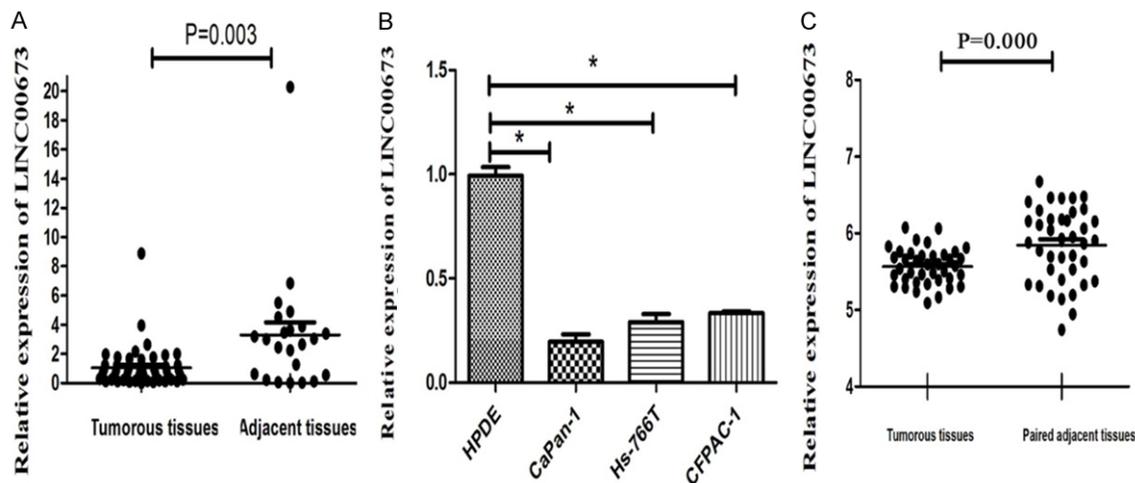


Figure 1. Expression levels of LINC00673 in pancreatic tissues and cell lines. A. LINC00673 was significantly downregulated in PDAC tissues compared with adjacent pancreatic tissues ($P = 0.003$; $n = 74$). GAPDH acted as an internal control. B. LINC00673 was significantly downregulated in three PDAC cell lines in comparison with HPDE cell line ($P \leq 0.001$). The results were presented as mean \pm SD for three experiments. *indicates $P \leq 0.001$. C. LINC00673 was significantly downregulated in PDAC tissues compared with corresponding adjacent tissues ($P = 0.000$; $n = 39$).

Table 1. The correlation of LINC00673 levels with clinicopathological characteristics in PDAC patients

Characteristic	Total	LINC00673 expression		P
		High	Low	
Age (years)				0.777
≥ 60	23	12	11	
< 60	27	13	14	
Gender				0.370
Female	17	7	10	
Male	33	18	15	
Diameter (cm)				0.037*
> 2 cm	33	13	20	
≤ 2 cm	17	12	5	
Differentiation				0.024*
Well and moderately	37	22	15	
Poorly	13	3	10	
T classification				0.355
T1+T2	15	9	6	
T3+T4	35	16	19	
Lymphatic metastasis				0.047*
Yes	12	3	9	
No	38	22	16	
Neural invasion				0.248
Yes	20	12	8	
No	30	13	17	

* $P < 0.05$.

addition, we used three cell lines of human PDAC and HPDE. We found that Hs-766T, CFPAC-1, and CaPan-1 were expressed with a low abundance ($P \leq 0.001$, **Figure 1B**).

Correlations between LINC00673 standards and characteristics of clinicopathology

The relative levels of LINC00673 were divided into two equal groups using the mean value as a standard. The associations between LINC00673 standards in PDAC tissues and parameters of clinicopathology of patients with pancreatic tumours are presented in **Table 1**. As shown, a low standard of LINC00673 displayed marked correlations with larger diameters ($P = 0.037$) and higher incidences of lymphatic metastases ($P = 0.047$) and poor differentiation ($P = 0.024$). However, there were no close correlations between LINC00673 levels and other clinicopathological parameters, including gender, age, T classification, and neural invasion ($P > 0.05$).

Using LINC00673 as a marker to observe the diagnostic value

In the curves of ROC, the imparities between tissues of PDAC and adjacent samples of pancreatic tissues were compared. The area under the curve (AUC) was 0.714 (95% confidence interval, 0.555-0.874; $P = 0.003$) (**Figure 2A**).

LINC00673 expression in PDAC

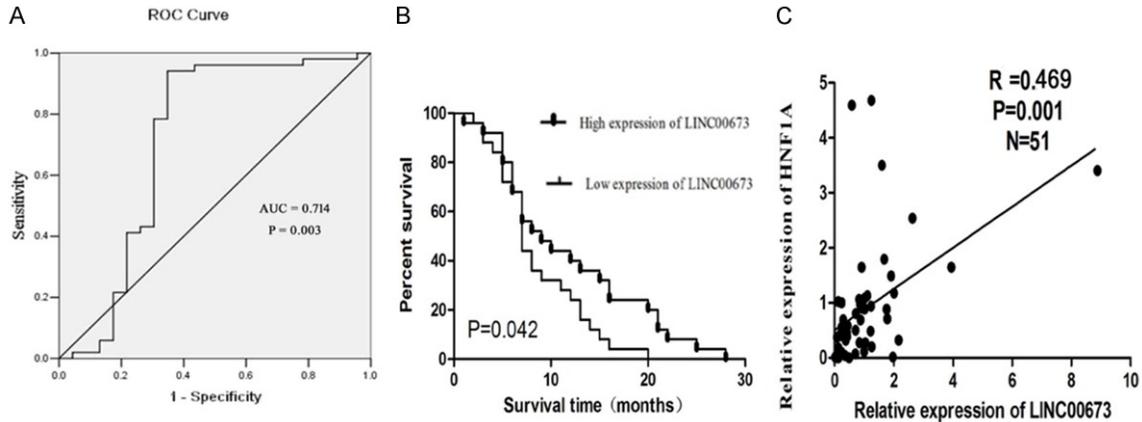


Figure 2. A. The curve of ROC was used to explore the potential diagnosis value of PDAC with LINC00673 as a bio-marker. The value of the AUC was 0.714 and the P -value was 0.003 ($n = 74$). B. LINC00673 was correlated with overall survival in patients with PDAC by Kaplan-Meier curve ($P = 0.042$; $n = 51$). Corresponding P analyzed by log-rank test. C. The expression level of HNF1A mRNA was positively correlated with LINC00673 in PDAC tissues (Pearson's correlation coefficient $r = 0.467$; $P = 0.001$; $n = 51$).

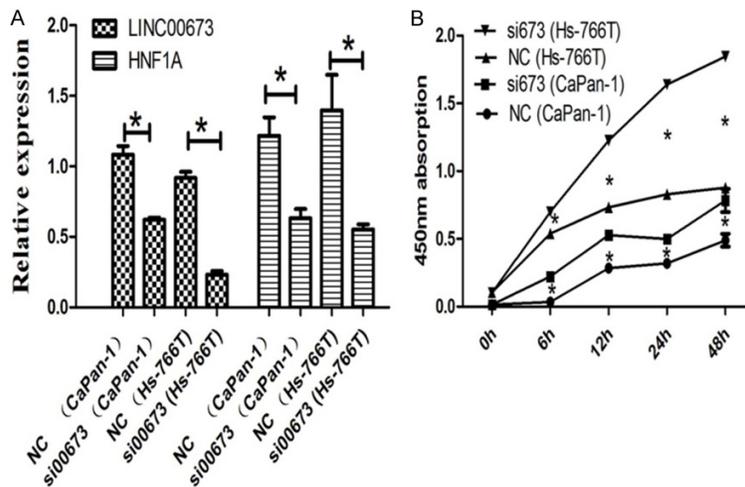


Figure 3. A. Analyses of qRT-PCR show that knockdown of LINC00673 could suppress expression levels of LINC00673 and HNF1A mRNA in CaPan-1 and Hs-766T cell line. *indicates $P < 0.05$. B. Compared to the negative control, transfection with the siRNA resulted in a significant increase in CaPan-1 and Hs-766T cell viability using CCK-8. *indicates $P < 0.05$.

Using LINC00673 as a marker to observe the prognosis value

Patients with low LINC00673 expression had a poor overall survival compared with the high LINC00673 group by Kaplan-Meier survival analysis ($P = 0.042$) (Figure 2B).

Knockdown of LINC00673 promoted PDAC cell proliferation via repression of HNF1A

To investigate the role of LINC00673 in PDAC, firstly, we examined the impact of knockdown

of LINC00673 in CaPan-1 and Hs-766T cells. As shown in Figure 3A, 48 h after transfection of the siRNA of LINC00673, qRT-PCR revealed that the expression of LINC00673 was significantly reduced in CaPan-1 and Hs-766T cells. After transfection, CCK-8 and EDU were conducted. Compared to the negative control, transfection with the siRNA resulted in a significant increase in CaPan-1 and Hs-766T cells viability and proliferation (Figures 3B, 4, 5). Next, results indicated that HNF1A mRNA was down-regulated in PDAC tissues and the expression level of HNF1A mRNA was positively correlated with LINC00673 in these

samples (Pearson's correlation coefficient $r = 0.469$; $P = 0.001$; Figure 2C). In addition, qRT-PCR analyses showed that knockdown of LINC00673 could suppress expression level of HNF1A mRNA (Figure 3A). Importantly, western blotting analyses showed that knockdown of LINC00673 could suppress expression level of HNF1A and activate expression levels of phospho-AKT (Ser473) and phospho-mTOR (Ser2448) (Figure 6). Taken together, these results showed that knockdown of LINC00673 could promote tumor growth of PDAC cells via repression of HNF1A.

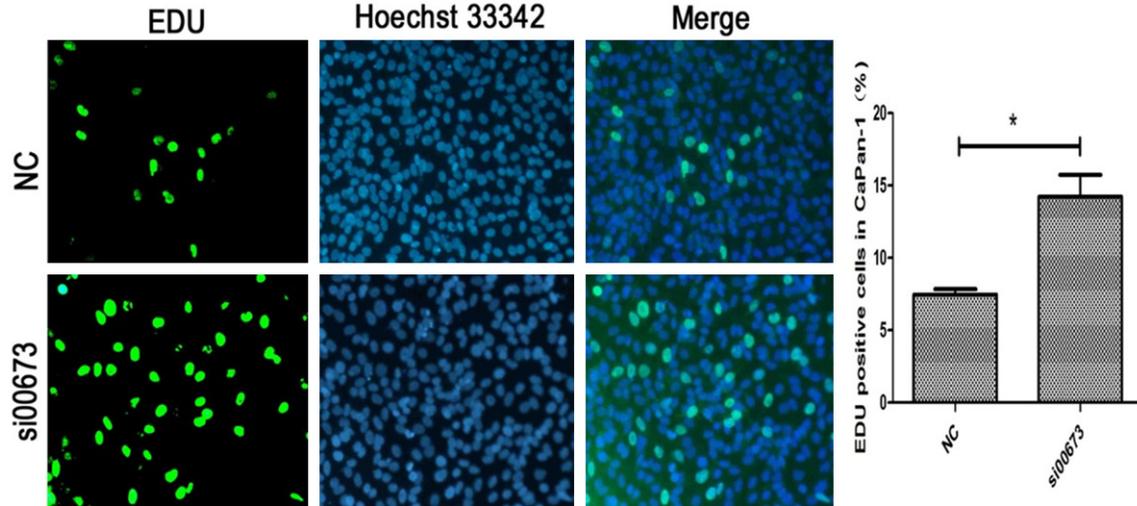


Figure 4. EDU (green)/Hoechst 33342 (blue) were used to confirm the results of CCK-8 assay in CaPan-1 cell line. The data represent the mean \pm SEM from three independent experiments. *P < 0.05.

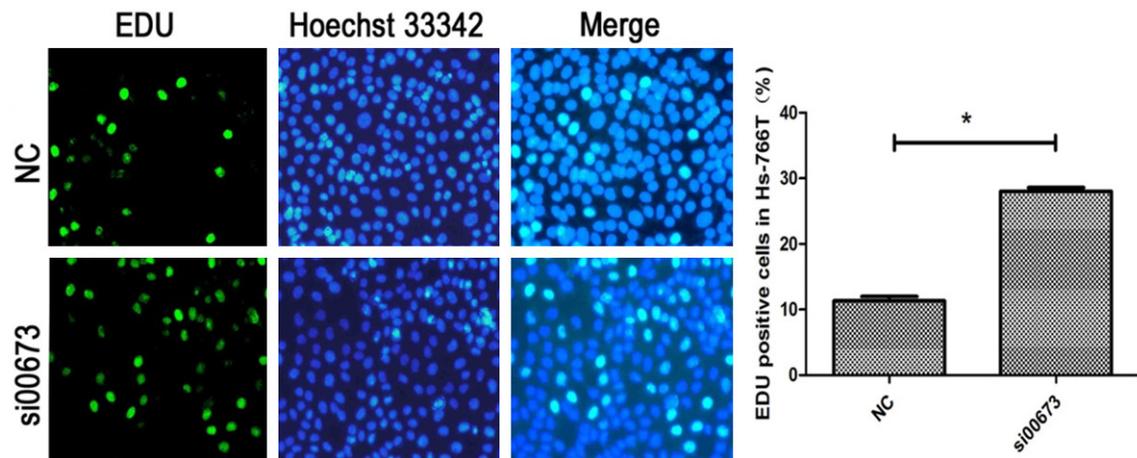


Figure 5. EDU (green)/Hoechst 33342 (blue) were used to confirm the results of CCK-8 assay in Hs-766T cell line. The data represent the mean \pm SEM from three independent experiments. *P < 0.05.

Discussion

Research has started to provide insight into the critical roles played by lncRNA in the various processes of cells, including tumorigenesis, development and differentiation [18]. For example, downregulated lncRNA LOC285194 is associated with liver metastasis, lymphatic metastasis and clinical stage in PDAC [19]. PC patients with upregulated lncRNA PVT1 have short overall survival times with PC [20]. High lncRNA MALAT-1 promotes cell invasion, migration and growth in PC [21-23]. HOTAIR is not a positive prognostic factor for PC patients and displays carcinogenic activity in both in vivo

and in vitro biological assays [24]. PDAC patients with upregulated lncRNA BC008363 have better overall survival times [25]. Upregulation of gas5 inhibits PC cell proliferation by targeting CDK6 [26]. H19 promotes the EMT of PC cells [27]. Upregulation of HULC is remarkably related to vascular invasion, lymphatic metastasis and large tumour size in PC [28]. Genome-wide screen identifies PVT1 as a regulator of Gemcitabine sensitivity in human pancreatic cancer cells [29]. By targeting OS-9 and HIF-1 α , ENST00000480739 inhibits tumorous cell invasion in PDAC [30]. However, the regulation of LINC00673 in the prognosis of PDAC patients is unknown.

LINC00673 expression in PDAC

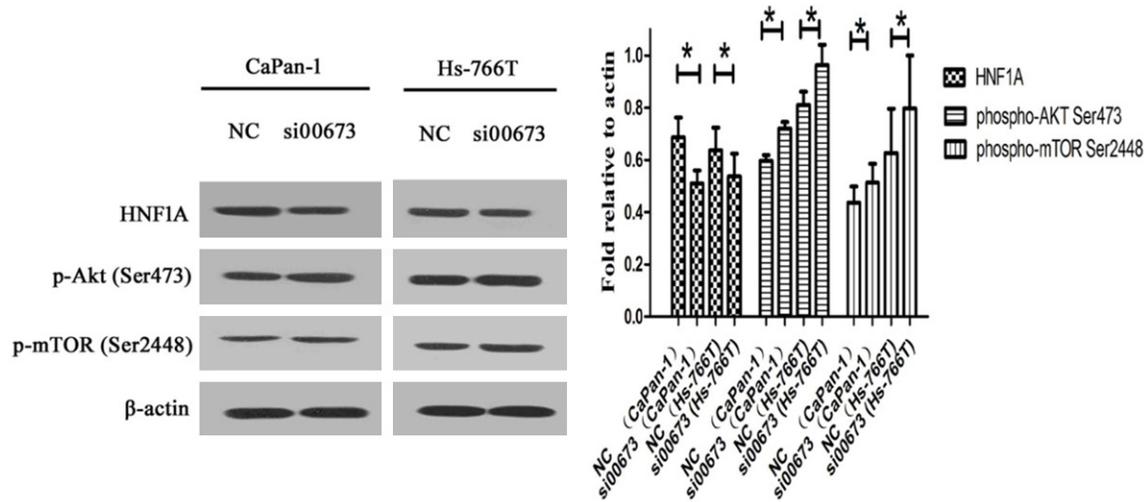


Figure 6. Effect of LINC00673 knockdown on AKT/mTOR signaling pathway was assessed by Western Blot. CaPan-1 and Hs-766T cells were transfected with control siRNA and one LINC00673 siRNA as indicated. 72 h after transfection, cell lysates were immunoblotted with anti-HNF1A antibody, anti-phospho-AKT Ser473 antibody, and anti-phospho-mTOR Ser2448 antibody. Membranes were stripped and re-probed with anti- β -actin antibody. The fold differences in protein expression levels of cells transfected with LINC00673 siRNA and control siRNA was presented as mean \pm SEM from three independent experiments. * $P < 0.05$.

It has shown that HNF1A expression is significantly decreased in PDAC patients in the paper of HNF1A as a possible tumor suppressor in pancreatic cancer [14]. Selective blocking of HNF1A by specific siRNA significantly promoted pancreatic cancer cell proliferation in vitro. They have further revealed that HNF1A knockdown activates Akt/mTOR signaling pathway in pancreatic cancer cell lines. These findings support a potential tumor suppressor role of HNF1A in PDAC [14]. The potential structure association of LINC00673 with HNF1A in PDAC is recently shown by the work of Childs EJ [9]. Our experiments indicate that the expression of HNF1A mRNA was positively correlated with LINC00673 expression in these PDAC tissues (Pearson's correlation coefficient $r = 0.469$; $P = 0.001$; **Figure 2C**). Importantly, qRT-PCR and western blotting analyses showed that knockdown of LINC00673 could suppress expression level of HNF1A (**Figures 3A, 6**). Taken together, these results showed that knockdown of LINC00673 promoted cell proliferation of PDAC via repression of HNF1A in vitro.

In our research, levels of LINC00673 were detected in pancreatic tissues and pancreatic cell lines by qRT-PCR. We found that the levels of LINC00673 were markedly downregulated in PDAC tissues compared with adjacent pancreatic tissues. Furthermore, compared to the

HPDE cell line, LINC00673 expression was significantly downregulated in three PDAC cell lines. Moreover, the standards of LINC00673 were significantly related to tumour diameter, differentiation and lymphatic metastasis and were not associated with age, gender, T classification and neural invasion. In addition, patients with low LINC00673 expression had a poor overall survival and knockdown of LINC00673 could obviously promote tumor growth of PDAC cells via repression of HNF1A, resulting in the activation of the mTOR signaling pathway, and afterwards, causing the proliferation of PDAC. These findings suggest that LINC00673 may play an important role in occurrence, growth or metastasis of PDAC. Therefore, we believe that LINC00673 might act as a potential tumor suppressor to predict occurrence, growth or metastasis.

In summary, we first identified that PDAC patients with low LINC00673 expression had a poor overall survival. LINC00673 expression was downregulated dramatically in PDAC tissues compared with adjacent pancreatic samples by qRT-PCR. The standard of LINC00673 was related to lymphatic metastasis, differentiation and tumour diameter. Knockdown of LINC00673 could promote tumor growth of PDAC cells via repression of HNF1A. Thus, LINC00673 might be applied as a potential tar-

get for therapy for PDAC patients. Furthermore, LINC00673 can serve as a potential tumor suppressor for the prognosis of PDAC patients. However, this research is limited by the small sample size. Furthermore, large-scale studies, plasma studies, studies compared with histological measure and studies of deep mechanism are warranted to confirm the above findings.

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Disclosure of conflict of interest

None.

Authors' contribution

PB designed the study, AGY and XDL carried out the experiments and AGY drafted the manuscript. YNW, HSD, YY, WL, YG, JXT and ABW took part in the statistical analysis. All authors read and approved the final manuscript. All procedures performed in studies involving human participants were in accordance with the ethical standards of the institutional and/or national research committee and with the 1964 Helsinki declaration and its later amendments or comparable ethical standards.

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