Linc-pint inhibits early stage pancreatic ductal adenocarcinoma growth through TGF-β pathway activation

HUIMIN LU, DUJIANG YANG, LING ZHANG, SHAN LU, JUN YE, MAO LI and WEIMING HU

Department of Pancreatic Surgery, West China Hospital, Sichuan University, Chengdu, Sichuan 610041, P.R. China

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Abstract. Long intergenic non-protein coding RNA, p53 induced transcript (Linc-pint) is a newly identified long non-coding RNA, which has demonstrated antitumor activities in various types of cancer. The present study aimed to investigate the role of Linc-pint in pancreatic ductal adenocarcinoma (PDAC). Plasma samples from patients with PDAC, and PDAC and normal cell lines were used in the study. Gene expression was analyzed by reverse transcription-quantitative polymerase chain reaction. Western blotting was used to assess protein level. Transforming growth factor β1 (TGF-β1) plasma level was determined by ELISA. Cancer cell proliferation was measured *in vitro* with the Cell Counting Kit-8 assy. The results demonstrated that Linc-pint plasma levels were significantly lower in patients with stage 0-1 PDAC compared with healthy controls. In addition, Linc-pint downregulation effectively distinguished patients with PDAC from healthy controls. Linc-pint and TGF-β1 plasma levels were positively correlated in patients with PDAC but not in healthy controls. Furthermore, Linc-pint overexpression upregulated TGF-β1 expression in PDAC cells but not in normal pancreatic ductal cells; however, exogenous TGF-β1 exhibited no significant effects on Linc-pint expression. Linc-pint overexpression and TGF-β1 both inhibited PDAC cell proliferation, whereas treatment with a TGF-β inhibitor reduced their inhibitory effects on cell proliferation. In conclusion, results from the present study suggested that Linc-pint may inhibit early stage PDAC growth through TGF-β pathway activation.

Introduction

Abnormally high cancer cell growth is the basis for cancer development and progression. Inhibiting tumor cell growth and division is therefore fundamental in the development of cancer therapies (1). Pancreatic cancer is a malignancy that develops from the pancreas and is responsible for >200,000 cases of mortality worldwide every year (2). Pancreatic ductal adenocarcinoma (PDAC) is the most common type of pancreatic cancer. Treatment outcomes for PDAC are generally poor due to the existence of distant tumor metastasis in 80-90% of patients at the time of diagnosis, since metastasis is a contraindication for surgical resection (3). Therefore, early tumor detection is crucial.

Transforming growth factor β (TGF-β) signaling exerts dual functions in cancer biology (4). For example, TGF-β signaling stimulates tumor metastasis through epithelial-mesenchymal transition (EMT) in the late stages of various types of cancer (5). However, TGF-β signaling activation in early stages of tumor growth inhibits tumor cell proliferation in most types of cancer, including PDAC (5,6). Therefore, TGF-β signaling pathway inhibition is considered a promising therapeutic target in pancreatic cancer (6). The crosstalk between TGF-β signaling and long non-coding RNAs (lncRNAs) has been reported in previous studies (7,8). Notably, long intergenic non-protein coding RNA, p53 induced transcript (Linc-pint) presents tumor suppressive potential in various types of cancer (9,10). A previous study reported that Linc-pint is decreased in plasma and tumor tissues of patients with pancreatic cancer, and that Linc-pint expression can distinguish patients with pancreatic cancer from healthy controls (9); however, the role of Linc-pint in pancreatic cancer development and progression remains unclear. Although Linc-pint is downregulated in numerous types of cancer, its roles and underlying molecular mechanisms of action in cancer biology remain unknown (10). In addition, Garitano-Trojaola *et al* (11) reported that Linc-pint is downregulated in acute lymphoblastic leukemia and participates in leukemic cell abnormal proliferation. Results from the present study suggested that Linc-pint may serve as a potential upstream activator of TGF-β signaling in PDAC.

Materials and methods

Subject enrollment and plasma preparation. A total of 206 patients with PDAC were diagnosed and treated at the West China Hospital (Chengdu, China) between January 2014 and January 2018. Among these patients, 46 were selected to serve as a patient group according to strict inclusion and exclusion criteria. The inclusion criteria were as follows: i) Patients diagnosed and treated for the first time, ii) patients

Correspondence to: Dr Weiming Hu, Department of Pancreatic Surgery, West China Hospital, Sichuan University, 37 Hao Guoxue Road, Chengdu, Sichuan 610041, P.R. China
E-mail: gkvks66@163.com

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diagnosed with PDAC through pathological examinations, iii) patients with stage 0-1 PDAC [according to the American Joint Committee on Cancer (AJCC) (12)] and iv) patients willing to participate. The exclusion criteria were as follows: i) Patients with other severe diseases, ii) patients who were treated prior to admission, iii) patients with chronic diseases, iv) patients with mental disorders and v) patients >70 years old. The patient group comprised 29 men and 17 women who were aged between 21 and 68 years (mean age, 45.5±6.1 years). Whole blood (10 ml) was extracted in the morning of the day following admission. Simultaneously, blood samples from 36 healthy volunteers who received routine physiological examination at the West China Hospital were collected and served as a control group. The control group included 23 men and 13 women who were aged between 23 and 66 years (mean age, 46.1±6.0 years). No significant differences in age and sex were found between the two groups. Blood samples were centrifuged in EDTA-containing tubes for 12 min at 1,200 x g and room temperature. Supernatants were collected as the plasma samples and stored in liquid nitrogen prior to use. The present study was approved by the Ethics Committee of West China Hospital. All subjects provided written informed consent.

Reverse transcription-quantitative polymerase chain reaction (RT-qPCR). Total RNA was isolated from plasma samples or cells with TRIzol® reagent (Invitrogen; Thermo Fisher Scientific, Inc., Waltham, MA, USA). For TGF-β1 treatment, cells were treated by exogenous TGF-β1 (Sigma-Aldrich; Merck KGaA, Darmstadt, Germany) dissolved in medium to 1, 10, 20 and 50 ng/ml for 24 h prior to RNA extraction. SuperScript III Reverse Transcriptase system (Thermo Fisher Scientific, Inc.) was used to synthesize cDNA according to the following conditions: 55˚C for 40 min and 75˚C for 15 min. RT-qPCR reactions were performed using SYBR® Green Real-Time PCR Master Mixes (Thermo Fisher Scientific, Inc.) as follows: 1 min at 95˚C, followed by 40 cycles at 95˚C for 20 sec and 45 sec at 58˚C. The following primers were used in PCR reactions: Linc-pint, forward 5'-CGTGGGAGC CCCTTTAAGTT-3', reverse 5'-GGGAGGTGGCGTAGTTTC TC-3'; and β-actin, forward 5'-GACCTCTATGCCAACACA GT-3' and reverse 5'-AGTACTTGCGCTCAGGAGGA-3'. The relative expression levels of Linc-pint were normalized to the endogenous control β-actin and were analyzed using the $2^{-\Delta\Delta Cq}$ method (13).

TGF-β1 detection by ELISA. TGF-β1 plasma levels were measured using a human TGF-β1 ELISA kit (cat. no. RAB0460; Sigma-Aldrich; Merck KGaA) according to the manufacturer's protocol.

Cell lines, cell culture and cell transfection. The human PDAC PL45 [cat. no. American Type Culture Collection (ATCC)® CRL-2558™] and normal human pancreatic ductal hTERT-HPNE (cat. no. ATCC® CRL-4023™) cell lines were purchased from ATCC (Manassas, VA, USA). Cells were cultured in ATCC-formulated Dulbecco's modified Eagle's medium (cat. no. 30-2002; ATCC) containing 10% fetal bovine serum (Sangon Biotech Co., Ltd., Shanghai, China) at 37˚C in a humidified incubator containing 5% CO_2.

PCR amplification was performed using Linc-pint primer with *Nhe*I cutting site at the 5'end to obtain full-length Linc-pint cDNA using Pfu DNA Polymerase kit (Promega Corporation, Madison, WI, USA). PCR product was subjected to agarose gel purification and was inserted into a linearized *Nhe*I-pEGFPC3 vector (Clontech Laboratories, Inc., Mountainview, CA, USA) to produce the Linc-pint expression vector. Cells from both cell lines were cultured for 8-12 h to reach 80-90% confluence. Cells ($5x10^5$) were then transfected with 10 nM vectors using Lipofectamine® 2000 reagent (cat. no. 11668-019; Invitrogen; Thermo Fisher Scientific, Inc.). Untransfected cells represented the control group. Cells transfected with empty vectors represented the negative control (NC) group. Experiments were performed 24 h following transfection.

Cell proliferation assay. Linc-pint expression was detected by RT-qPCR 24 h post-transfection. Cell Counting Kit-8 (CCK-8; Sigma-Aldrich; Merck KGaA) was used to detect cell proliferation when the Linc-pint overexpression rate reached >200% compared with control cells. A $5x10^4$ cells/ml cell suspension was prepared and a 100-μl cell suspension containing $5x10^3$ cells was seeded into a 96-well plate. For TGF-β1 treatment, cells were treated with exogenous TGF-β1 for 24 h at 37˚C, prior to seeding into the 96-well plate. For TGF-β inhibitor treatment, cells were treated with 10 ng/ml SD 208, which is a potent ATP-competitive TGF-β receptor inhibitor (R&D Systems, Inc., Minneapolis, MN, USA) for 24 h at 37˚C, prior to seeding into the 96-well plate. The plate was placed in the incubator, and CCK-8 solution (10 μl) was added after 12, 24, 48, 72 and 96 h for 4 h at 37˚C. Optical density was measured at 450 nm using a microplate reader (Bio-Rad Laboratories, Inc., Hercules, CA, USA).

Western blotting. Cells were lysed using radioimmunoprecipitation assay buffer (Thermo Fisher Scientific, Inc.) and the protein concentration was measured using a bicinchoninic acid assay kit according to the manufacturers' protocol. Proteins (20 μg) were separated by 10% SDS-PAGE and transferred onto polyvinylidene difluoride membranes. The membranes were blocked in 5% skimmed milk for 1 h at room temperature and incubated with rabbit primary antibodies against TGF-β1 (cat. no. ab92486; 1:1,500; Abcam, Cambridge, MA, USA) and GAPDH (cat. no. ab9485; 1:1,000; Abcam) overnight at 4˚C. Membranes were then incubated with horseradish peroxidase-conjugated immunoglobulin G secondary antibody (cat. no. MBS674747; 1:1,000; MyBioSource, Inc., San Diego, CA, USA) for 2.5 h at room temperature. Signals were detected using enhanced chemiluminescence reagent (Sigma-Aldrich; Merck KGaA). Data were analyzed via densitometry using ImageJ software v1.6 (National Institutes of Health, Bethesda, MD, USA).

Statistical analysis. Statistical analyses were performed using GraphPad Prism 6.0 software (GraphPad Software, Inc., La Jolla, CA, USA). Gene expression and cell proliferation data were expressed as the means ± standard deviation. Data comparisons were performed by Student's t-test (comparison between two groups) or one-way analysis of variance followed by least-significant difference test (comparison between multiple groups). The association between Linc-pint plasma

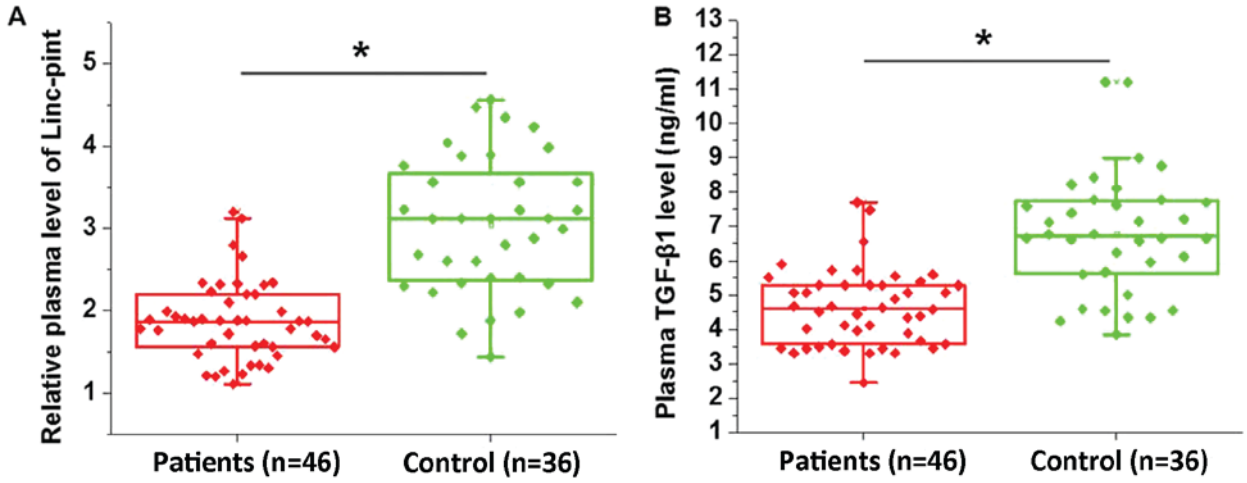

Figure 1. Linc-pint and TGF-β1 plasma levels are significantly lower in patients with early stage PDAC. Comparison of plasma levels of Linc-pint (reverse transcription-quantitative polymerase chain reaction) and TGF-β1 (ELISA) between two groups revealed significantly lower plasma levels of (A) Linc-pint and (B) TGF-β1 in patients with PDAC compared with in healthy controls. Experiments were performed in triplicate. *P<0.05. Linc-pint, long intergenic non-protein coding RNA, p53 induced transcript; PDAC, pancreatic ductal adenocarcinoma; TGF-β1, transforming growth factor β1.

levels and the clinical characteristics of patients with PDAC was analyzed by χ^2 test. Pearson correlation coefficient was used for correlation analysis. Receiver operating characteristic (ROC) curves and area under the curve (AUC) were used to assess the diagnostic value of plasma Linc-pint in patients with PDAC. P<0.05 was considered to indicate a statistically significant difference.

Results

Plasma levels of Linc-pint and TGF-β1 are significantly lower in patients with early stage PDAC. Plasma levels of Linc-pint and TGF-β1 in patients with PDAC and healthy controls were measured by RT-qPCR and ELISA, respectively. As presented in Fig. 1, significantly lower plasma levels of Linc-pint (Fig. 1A) and TGF-β1 (Fig. 1B) were detected in patients with PDAC compared with in healthy controls (P<0.05).

Downregulation of Linc-pint plasma levels distinguishes patients with PDAC from healthy controls. ROC analysis was performed to evaluate the diagnostic value of plasma Linc-pint in patients with PDAC. As presented in Fig. 2, AUC was 0.8934, with a standard error of 0.03646 and 95% confidence interval of 0.8219-0.9649 (P<0.001). AUC >0.65 indicated potential diagnostic values.

Plasma levels of Linc-pint and TGF-β1 are positively correlated in patients with PDAC but not in healthy controls. Pearson correlation coefficient was used to assess the correlation between plasma Linc-pint and TGF-β1 levels. As presented in Fig. 3A, Linc-pint and TGF-β1 plasma levels were positively correlated in patients with PDAC (P<0.0001). Conversely, no correlation between plasma levels of Linc-pint and TGF-β1 was detected in healthy controls (P=0.1289).

Plasma levels of Linc-pint are significantly associated with tumor size in patients with PDAC. Patients with PDAC were divided into high (n=23) and low (n=23) expression groups according to the median plasma level of Linc-pint. The

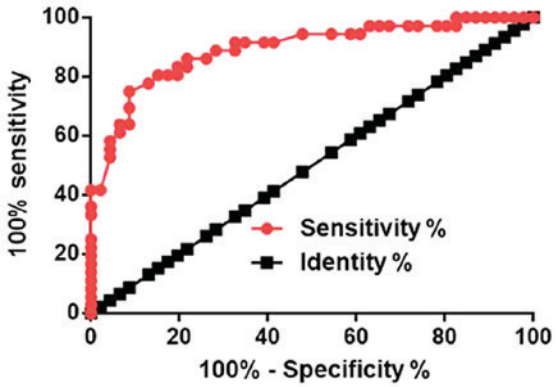

Figure 2. ROC analysis of the diagnostic value of Linc-pint plasma levels in PDAC. ROC curve analysis was performed to evaluate the diagnostic value of plasma Linc-pint for PDAC. Results revealed that downregulation of Linc-pint plasma levels distinguished patients with PDAC from healthy controls. Linc-pint, long intergenic non-protein coding RNA, p53 induced transcript; PDAC, pancreatic ductal adenocarcinoma; ROC, receiver operating curve.

association between Linc-pint plasma levels and clinical characteristics of patients with PDAC was analyzed by χ^2 test. As presented in Table I, plasma levels of Linc-pint were significantly associated with tumor size (P<0.01), but not with age, sex, and drinking and smoking habits (P>0.05).

Linc-pint overexpression upregulates TGF-β1 in PDAC cells. Cells overexpressing Linc-pint were generated to further investigate the interactions between Linc-pint and TGF-β1. As presented in Fig. 4A, Linc-pint overexpression was reached in PL45 and hTERT-HPNE cell lines compared with control and NC cells. In addition, PL45 cells overexpressing Linc-pint exhibited significantly upregulated TGF-β1 expression (P<0.05). However, Linc-pint overexpression had no significant effects on TGF-β1 in hTERT-HPNE cells (P>0.05). Conversely, treatment with exogenous TGF-β1 at 1, 10, 20 and 50 ng/ml for 24 h had no significant effects on Linc-pint expression in both cell lines (Fig. 4B).

Table I. Association between long intergenic non-protein coding RNA, p53 induced transcript plasma levels and clinicopathological characteristics of patients with pancreatic ductal adenocarcinoma.

Variable	Cases	High-expression	Low-expression	χ^2	P-value
Age (years)					0.37
≥45	25	11	14	0.79	
<45	21	12	9		
Sex					0.13
Male	29	12	17	2.33	
Female	17	11	6		
Primary tumor diameter (cm)					<0.01
≥2	28	9	19	9.12	
<2	18	14	4		
Smoking					0.38
Yes	23	10	13	0.78	
No	23	13	10		
Drinking					0.35
Yes	31	14	17	0.89	
No	15	9	6		

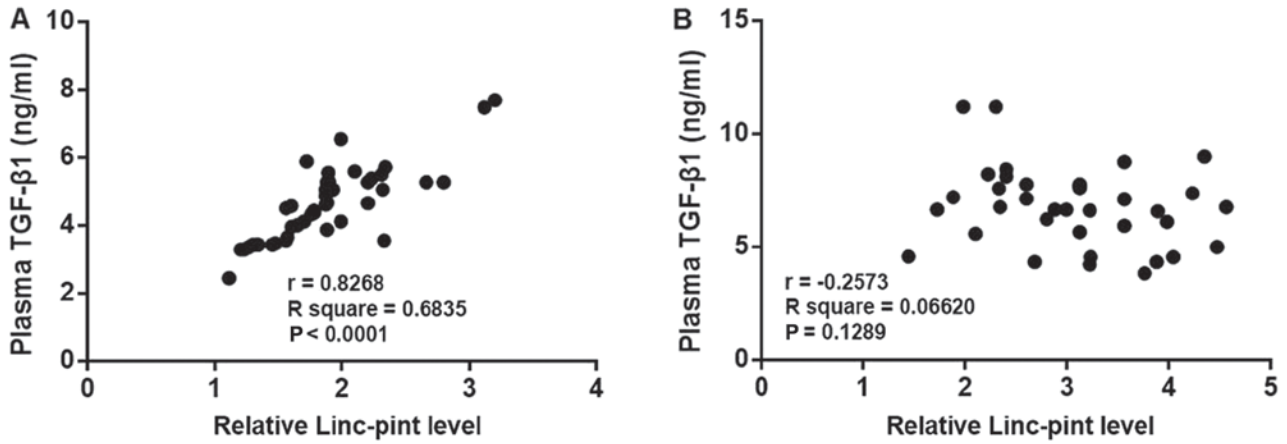

Figure 3. Linc-pint and TGF-β1 plasma levels are positively correlated in (A) patients with pancreatic ductal adenocarcinoma but not in (B) healthy controls (Pearson correlation coefficient). Linc-pint, long intergenic non-protein coding RNA, p53 induced transcript; TGF-β1, transforming growth factor β1.

Linc-pint overexpression and treatment with exogenous TGF-β1 inhibit PDAC cell proliferation. CCK-8 assay was performed to detect cell proliferation under different conditions. Linc-pint overexpression or treatment with exogenous TGF-β1 significantly inhibited PL45 cell proliferation compared with control and NC cells (P<0.05; Fig. 5A). In addition, treatment with SD 208 at 10 ng/ml significantly increased PDAC cell proliferation and reduced the inhibitory effects of Linc-pint overexpression and exogenous TGF-β1 treatment on cell proliferation (P<0.05; Fig. 5A). However, none of the aforementioned treatments had significant effect on hTERT-HPNE cell proliferation (P>0.05; Fig. 5B).

Discussion

Although Linc-pint has been described as a tumor suppressor in various types of cancer, the underlying mechanisms of action of Linc-pint are mainly based on *in vitro* cell experiments, whereas clinical findings are lacking. The present study demonstrated that Linc-pint may be a tumor suppressive lncRNA in PDAC and may possess diagnostic value. In addition, results suggested that Linc-pint action may be associated with TGF-β1 upregulation.

In most types of cancer, TGF-β signaling activation inhibits tumor cell growth at early stages of tumor development but promotes tumor metastasis at late stages (14). A previous study reported that TGF-β signaling has a tumor suppressive role during pancreatic cancer initiation (15). To further confirm the role of TGF-β in pancreatic cancer, the present study only included patients with PDAC at AJCC stage 0 and 1, where the tumor had not spread to nearby lymph nodes or distant sites. Results demonstrated that TGF-β1 plasma levels were significantly downregulated in patients with PDAC compared with in healthy controls. In

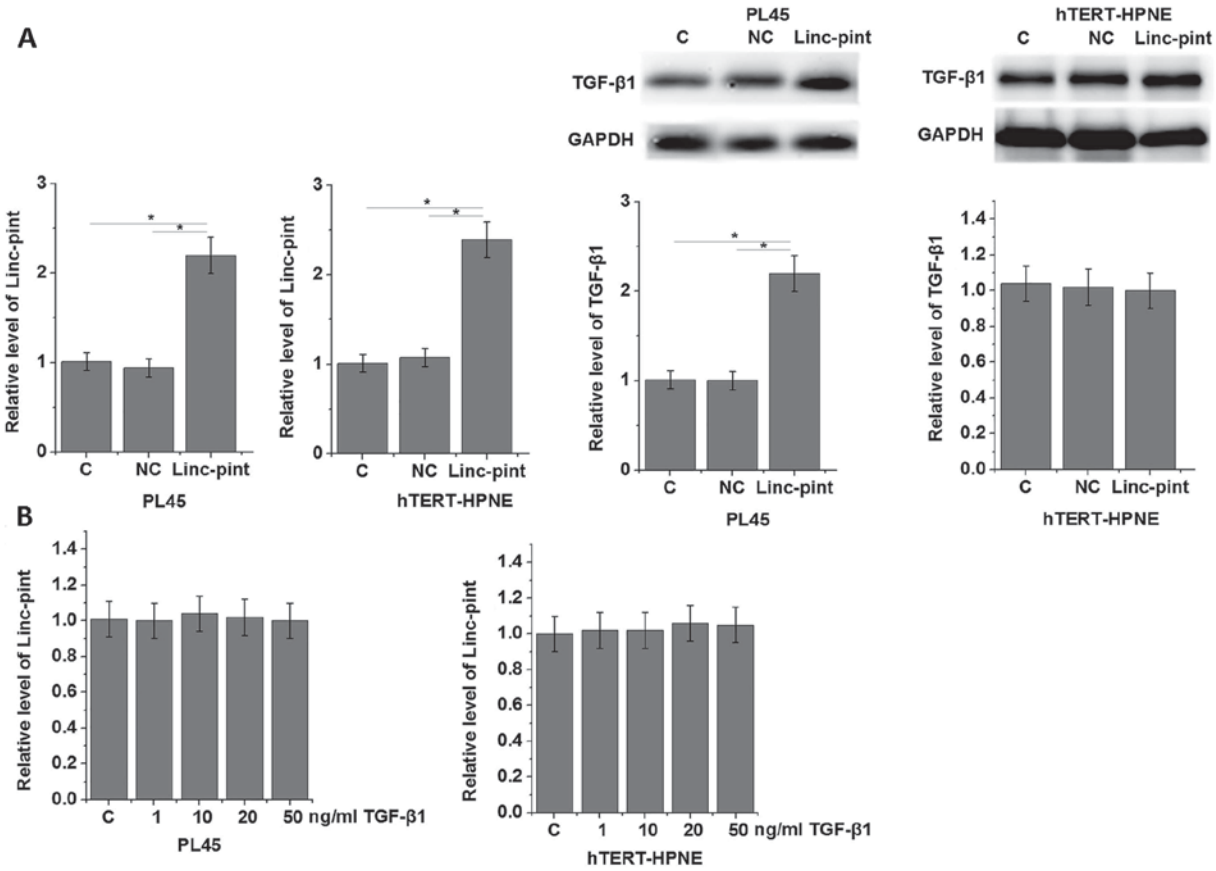

Figure 4. Linc-pint overexpression upregulates TGF-β1 in PDAC cells. (A) Western blotting revealed that Linc-pint overexpression upregulated TGF-β1 in PDAC cells but not in normal pancreatic ductal cells. (B) Reverse transcription-quantitative polymerase chain reaction demonstrated that treatment with exogenous TGF-β1 had no significant effect on Linc-pint expression levels in both cell lines. Experiments were performed three times. *P<0.05. C, control; Linc-pint, long intergenic non-protein coding RNA, p53 induced transcript; NC, negative control; PDAC, pancreatic ductal adenocarcinoma; TGF-β1, transforming growth factor β1.

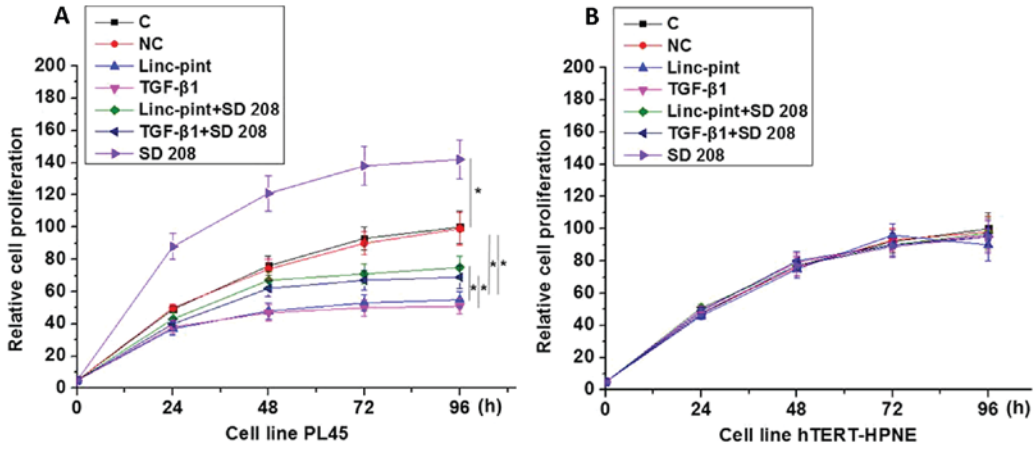

Figure 5. PL45 and hTERT-HPNE cell proliferation under various conditions. (A) Cell Counting Kit-8 assay demonstrated that Linc-pint overexpression and treatment with exogenous TGF-β1 significantly inhibited cancer cell proliferation. Treatment with SD 208 significantly increased pancreatic ductal adenocarcinoma cell proliferation and decreased the inhibitory effects of Linc-pint overexpression and exogenous TGF-β1 treatment on cell proliferation. (B) No significant differences in the hTERT-HPNE cell proliferation were observed after similar treatments. This experiment was performed three times. *P<0.05. C, control; Linc-pint, long intergenic non-protein coding RNA, p53 induced transcript; NC, negative control; SD 208, potent ATP-competitive TGF-βRI inhibitor; TGF-β1, transforming growth factor β1.

addition, treatment with exogenous TGF-β1 inhibited PDAC cell proliferation. These findings suggested that TGF-β signaling may have a tumor suppressive role during PDAC initiation.

Linc-pint downregulation has been reported in various types of human cancer cell lines (10). A recent study reported that Linc-pint is downregulated in plasma and tumor tissues of patients with pancreatic cancer, of which

80% have PDAC (9). Similarly, results from the present study demonstrated that Linc-pint plasma levels were decreased in patients with PDAC compared with in healthy controls, which suggested that Linc-pint may act as a tumor suppressor in PDAC. In addition, TGF-β signaling can interact with lncRNAs to participate in physiological and pathological processes (7,8). For example, certain lncRNAs, including plasmacytoma variant translocation 1, promote EMT through TGF-β signaling to accelerate tumor metastasis in pancreatic cancer (16). However, the association between lncRNAs and TGF-β signaling in human PDAC growth remains unknown. In the present study, a positive correlation between Linc-pint and TGF-β1 plasma levels was observed in patients with PDAC. In addition, Linc-pint overexpression promoted TGF-β1 expression in PDAC cells, but treatment with TGF-β1 failed to affect Linc-pint expression. Furthermore, treatment with a TGF-β inhibitor reduced the inhibitory effects of Linc-pint overexpression on PDAC cell proliferation. These results suggested that Linc-pint may be an upstream activator of TGF-β1, which could inhibit PDAC growth. However, since there was no correlation between Linc-pint and TGF-β1 plasma levels in healthy controls, and because Linc-pint overexpression had no effect on TGF-β1 expression in normal pancreatic ductal cells, the regulatory effects of Linc-pint on TGF-β1 expression may be mediated by other disease factors.

A recent study reported that Linc-pint downregulation has diagnostic and prognostic values in patients with pancreatic cancer at different pathological stages (9). Conversely, the present study investigated the role of Linc-pint in tumor growth in early stages PDAC, and revealed the molecular mechanism underlying Linc-pint involvement in PDAC. Results from the present study demonstrated that Linc-pint plasma level were not associated with age, sex, and smoking and drinking habits, although these characteristics were reported to affect the expression of certain lncRNAs (17-21). These findings suggested that Linc-pint may serve as a reliable diagnostic marker for patients with early stage PDAC.

A potential limitation of the present study is that Linc-pint function was not investigated in an animal model of PDAC. However, *in vivo* experiments will be performed in future investigations to further confirm the findings from the present study. In addition, TGF-β has been reported to be a key regulator in proliferation of multiple, but not all types, of cells (22). In the present study, the hTERT-HPNE cell line exhibited no significant difference in cell proliferation following treatment with increasing doses of TGF-β1, which suggested that TGF-β signaling may not be a regulator of hTERT-HPNE cells.

In conclusion, Linc-pint and TGF-β1 were downregulated in early stage PDAC, and this study suggested that Linc-pint may inhibit PDAC growth by activating the TGF-β pathway.

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Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Authors' contributions

HL and WH designed the experiments. HL, DY and LZ performed the experiments. SL, JY and ML analyzed data. WH drafted manuscript. All authors read and approved the final manuscript.

Ethics approval and consent to participate

This study has been approved by the Ethics Committee of West China Hospital. All patients provided informed consent.

Patients consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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