Mutant p53-driven IncRNA LINC00857 promotes pancreatic cancer metastasis through OTUB1-mediated FOXM1 deubiquitination

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Abstract

Background

p53 mutations are considered to be the second most common type of mutation in PC, and significantly promote its metastasis. However, the underlying molecular mechanisms, especially the regulatory relationship with long noncoding RNAs (lncRNAs) remain unclear.

Methods

Based on TCGA and GTEx databases and integrating bioinformatics analyses, the overexpression of lncRNA LINC00857 in PC tissues was detected and further validated in a cohort of PC tissues. The effects of LINC00857 on EMT, migration and invasion were determined by in vitro and in vivo experiments. RNA pull-down, RNA immunoprecipitation (RIP), chromatin isolation by RNA purification (ChIRP) and fluorescence in situ hybridization (FISH) were carried out to reveal the interaction between LINC00857 and FOXM1, OTUB1. Chromatin immunoprecipitation (ChIP) and Luciferase reporter assay were used to evaluate the regulatory effect of mutant p53 on LINC00857.

Results

lncRNA LINC00857 exhibited a significantly elevated level in PC and was involved in poor prognosis, and its expression was significantly upregulated in the p53 mutant group compared with the p53 wild-type group. Moreover, LINC00857 remarkably suppressed tumour metastasis in vitro and in vivo in a FOXM1 dependent manner. Mechanistically, LINC00857 simultaneously bound to FOXM1 and the deubiquitinase OTUB1, serving as a protein scaffold to enhance the interaction between FOXM1 and OTUB1, which inhibited FOXM1 degradation through the ubiquitin–proteasome pathway. Interestingly, we found that mutant p53 promoted the transcription of LINC00857 by binding to its promoter region. Finally, atorvastatin, a commonly applied lipid-lowering drug in the clinic, was suggested to inhibit PC metastasis by inhibiting the mutant p53-LINC00857 axis.

Conclusions

Taken together, our results uncovered new insights into the biology driving PC metastasis and indicated that the mutant p53-LINC00857 axis might serve as a novel therapeutic target for PC metastasis.

Background

Pancreatic cancer (PC) is one of the most lethal diseases in developed countries, with a five-year survival rate of less than 10%, and it is expected to become the second leading cause of cancer death in 2030[1, 2]. PC is characterized by insidious onset, rapid development, high rates of metastasis and recurrence,
metastasis is still the leading cause of death in PC patients [3]. PC cells exhibit high metastatic potential, invade locally and migrate to secondary organ sites to form metastatic niches [4]. Thus, it is important to identify the underlying molecular mechanisms of PC metastasis and develop new therapeutic targets.

Many PCs are characterized by sequential accumulation of alterations in the oncogene KRAS and loss/mutation of the tumour suppressors CDKN2A, TP53, and/or SMAD4[5]. The tumour suppressor gene TP53 is also frequently mutated in human PC (50–75%), predominantly exhibiting missense mutations, and studies have suggested that mutant p53 promotes tumour progression, which includes metastasis [6]. Considering the robust transcript function of mutant p53 in cancer development, it is reasonable to speculate that mutant p53 may change its biology behavior. Studies have shown that mutated p53 activates numerous downstream gene networks, thereby promoting the metastasis of PC. For example, mutant p53 potentiates transcriptional induction of several heat shock proteins by enhancing HSF1 stabilization and activation and participating in HSF1 transcriptional complexes at Hsp gene promoters [7]. Additionally, a study showed that circPVT1 expression was transcriptionally enhanced by the mut-p53/YAP/TEAD complex [8]. Hence, among these mechanisms, long noncoding RNAs (lncRNAs) can also function as downstream genes to perform related functions [9]. However, the comprehensive mutant p53-regulated lncRNA network in PC metastasis remains to be investigated.

LncRNAs are noncoding RNAs that are longer than 200 nucleotides and usually exhibit loss of protein-coding potential [10]. Accumulating studies have shown that IncRNAs are essential regulators of almost every aspect of the metastatic capacity of cancer cells [11]. Moreover, IncRNAs perform diverse biological functions, such as regulating protein and RNA stability, modulating transcription, guiding protein–DNA interactions and driving protein scaffold formation. Yang et al. revealed that lncRNA SLC26A-AS1 suppressed thyroid cancer metastasis by promoting the interaction between DDX5 and the E3 ligase TRIM25, which promoted DDX5 degradation through the ubiquitin–proteasome pathway [8]. Although studies on the relationship between lncRNAs and tumour metastasis are increasing, the molecular mechanisms linking lncRNAs to PC metastasis remain elusive.

In this study, we identified an oncogenic lncRNA, termed LINC00857, which was highly expressed in PC tissues, and determined that overexpression of LINC00857 was driven in part by mutant p53. Moreover, the results showed that LINC00857 promoted the metastasis of PC both in vitro and in vivo, and the promoting effect of LINC00857 on PC metastasis was partially dependent on Forkhead Box M1 (FOXM1). We found that LINC00857 could play a role as a protein scaffold by promoting the interaction between OTU domain-containing ubiquitin aldehyde-binding protein 1 (OTUB1) and FOXM1, which inhibited FOXM1 degradation through the ubiquitin–proteasome pathway. Finally, we revealed that the promoting effect of mutant p53 on LINC00857 is mediated by binding to the promoter region, thus promoting transcription, and found that the mutant p53-LINC00857 axis in PC can be inhibited by the common clinical medication atorvastatin.

2 Materials And Methods
2.1 Cell culture and treatment

The pancreatic cancer cell lines Panc-1 and MIA PaCa-2 were purchased from the Chinese Academy of Sciences Cell Bank of Type Culture Collection (CBTCCCAS; Shanghai, China). Panc-1 and MIA PaCa-2 cells were cultured in Dulbecco's modified Eagle's medium (DMEM; Gibco; Thermo Fisher Scientific, USA). Atorvastatin (ATOR; purity ~ 99%) was purchased from MedChemExpress (MCE, USA) and dissolved in dimethyl sulfoxide (DMSO). For IC50 assays, cells were cultured at a density of 5 000 per well in 96-well plates. The cells were treated with the corresponding concentrations of drug and cultured for 48 h. A Cell Counting Kit-8 (Sangon Biotech, Shanghai, China) was used to measure the effects of the drug by measurement of the absorbance at 450 nm using a microplate reader (Thermo Fisher Scientific, USA) after incubation at 37°C for an additional 2–4 h.

2.2 Fluorescence in situ hybridization (FISH)

The biotin-labelled RNA probes for LINC00857 were produced using the h-LINC00857 FISH Probe Mix (Ribobio, China). Cells were plated into 24-well plates and then fixed with paraformaldehyde when they grew to an appropriate density. After fixation, hybridization was carried out, and the LINC00857 probe mix was added and incubated overnight. The next day, DAPI staining was performed in the dark after washing, and observation was performed under a fluorescence microscope (Nikon A1R/A1).

2.3 Transwell and wound healing assays

For the Transwell migration assay, cells in 200 µl of serum-free DMEM were added to the upper chambers containing 8-µM pore polycarbonate membrane filters (Millipore, USA). For the invasion assay, 8x10⁴ cells in 200 µl of serum-free DMEM were added to the upper chamber inserts containing membranes that had been coated with Matrigel (BD Biosciences, USA) 4 h in advance. Medium containing 20% FBS (700 µl) was added to the lower chambers. After 24 or 48 h, Panc-1 or MIA Paca-2 cells that had migrated to the lower chambers through the pores were stained with 0.2% crystal violet solution and counted. For the wound healing assays, cells in different groups were seeded in 6-well plates. When the cells reached 100% confluence, a 200 µl pipette tip was used to scratch the artificial wounds in the monolayer and, wound healing was observed by inverted microscopy after 24 h.

2.4 RNA pull-down and RNA-binding protein immunoprecipitation (RIP) assays

Sense and antisense LINC00857 RNAs were designed and synthesized by BersinBio Biological Co., Ltd. After a sufficient number of cells were collected, the RNA pull-down assay was carried out in accordance by using an RNA Pull-Down Kit (BersinBio, Guangzhou, China) according to the manufacturer’s instructions. The retrieved proteins were then analysed by liquid chromatography–tandem mass spectrometry (LC–MS/MS) (PTM Biolabs, Hangzhou, China) and western blotting. RIP assays were performed with a RIP Kit (BersinBio, Guangzhou, China) with anti-FOXM1 and anti-OTUB1 antibodies or IgG isotype control. qRT–PCR was carried out to detect coprecipitated RNAs.
2.5 Co-immunoprecipitation (Co-IP)

Co-IP between FOXM1 and OTUB1 was performed. Cells were lysed in NP-40 lysis buffer (P0013F, Beyotime, China) at 4°C for 30 min. Cell lysates were incubated with antibodies against FOXM1 or OTUB1 or with control IgG overnight at 4°C and with Dynabeads® Protein G beads (Invitrogen) the next day for 2 h at 4°C. The beads were washed five times with NP-40 lysis buffer, and the results were verified by western blotting.

2.6 Chromatin immunoprecipitation (ChIP) and dual luciferase reporter assay

ChIP was performed using a SimpleChIP Enzymatic Chromatin IP Kit (Cell Signaling Technology). Pancreatic cancer cells were seeded in 10 cm dishes, crosslinked with the reagent when they were 90% confluent, and lysed with SDS buffer followed by ultrasonication. Then, ultrasonication was used to fragment the DNA into pieces of 100–500 bp, and specific antibodies or normal mouse IgG were used to pull down the DNA. After washing with high-salt and low-salt buffers, DNA was eluted and crosslinking was reversed, and enriched sequences were examined using qPCR. For the dual luciferase reporter assay, the promoter region containing only the WT1 and WT2 sequences and the promoter region containing only the MUT1 and MUT2 sequences were synthesized and cloned into the pmirGLO basic luciferase reporter vector (Miaolingbio, China). Luciferase activity was measured with a Dual Luciferase Assay system (Beyotime, Shanghai, China). Renilla luciferase activity was normalized to firefly luciferase activity.

2.7 Chromatin isolation by RNA purification (ChIRP)

ChIRP was performed using a ChIRP RNA Interactome Kit (BersinBio, Guangzhou, China) according to the manufacturer’s instructions. A biotinylated tiling probe targeting LINC00857 was synthesized and provided by RiboBio. Before crosslinking, pancreatic cancer cells were grown in DMEM supplemented with 10% foetal bovine serum at 37°C in a humidified 5% CO2 atmosphere. Cell lysates were harvested after incubation for 24 h at a confluence of 80%. Cells were crosslinked with 1% fresh formaldehyde and sonicated for hybridization. After chromatin was sheared into 100–500 bp fragments, cell lysates were incubated with the different biotinylated DNA probe solutions at 4°C overnight. The binding complexes were coated with streptavidin-conjugated magnetic beads. Proteins were finally eluted and purified from the magnetic beads for western blot analysis. An additional sample was digested with 15 µg/ml RNase for 10 min at room temperature before incubation with the LacZ probe for use as a negative control.

2.8 Immunofluorescence (IF) assay

After culture in 24-well plates overnight, pancreatic cells were washed with PBS three times and then fixed in 4% paraformaldehyde for 20 min. Then, cells were permeabilized with 0.5% Triton X-100 (Sigma–Aldrich), blocked with 1% BSA (Sigma–Aldrich) for 1 h and incubated with the appropriate primary antibodies overnight followed by staining with CoraLite 488-conjugated or CoraLite 594-conjugated
secondary antibodies at 25°C for 1 h. After cells were washed with PBS three times, nuclei were stained with 4,6-diamidino-2-phenylindole (DAPI) (Sigma–Aldrich). Images were visualized and acquired with a Nikon A1R/A1 microscope (Nikon, Japan).

2.9 Tail vein injection model

BALB/c female nude mice aged 4 weeks were purchased from Chengdu Gembio Co., Ltd (Chengdu, China) and maintained in a specific pathologic-free environment. Mice were randomly divided into three groups with four mice in each group, and KPC cells (immortalized cells from the PC of a KPC mouse) were stably transduced with LINC00857 overexpression or negative control luciferase-labelled no-load plasmids using a lentiviral system and were then injected into the mice. Three weeks later, the mice were intraperitoneally injected with D-Luc and then evaluated with a small animal imager (IVIS Lumina LT, USA) for metastasis, and their lungs were surgically excised, photographed, and measured. Subsequently, lung tissues were fixed with 10% formalin and embedded in paraffin for haematoxylin and eosin (H&E) staining for histological confirmation. All animal procedures were performed in compliance with ethical standards and approved by the Animal Care Committee of Xi’an Jiaotong University.

3 Results

3.1 LINC00857 is upregulated in PC and associated with poor prognosis.

To identify lncRNAs involved in the progression and metastasis of PC, we compared the expression of lncRNAs in PC and normal samples from TCGA (The Cancer Genome Atlas, https://cancergenome.nih.gov/) and GTEx (Genotype-Tissue Expression, https://commonfund.nih.gov/GTex) databases. The differential gene expression between them is shown in volcano plots and a heatmap (|log2FoldChange| >1, P < 0.05; Fig. 1A, Fig. S1A). One representative lncRNA, LINC00857, was considered to have abnormal upregulation.

Through Coding Potential Calculator analysis, LINC00857 had a very low protein-coding ability (Supplementary Fig. S1B-E), which means that LINC00857 was indeed a long noncoding RNA. After extracting and comparing the expression data of LINC00857 from the TCGA and GTEx databases, we found that LINC00857 was significantly overexpressed in PC tissues (Fig. 1B), and this result was further confirmed in our centre (Fig. 1C). Moreover, TCGA data showed that LINC00857 expression levels were significantly associated with overall survival (OS) and progression-free survival (PFS) in PC patients (Fig. 1D, E). To verify the accuracy of LINC00857 expression for predicting prognosis, a ROC curve was constructed, and the results suggested that the area under the curve (AUC) of LINC00857 was 0.6238, which showed a good ability to predict prognosis. Subsequently, through PCR verification after nuclear-cytoplasmic separation, we found that LINC00857 was localized in both the nucleolus and cytoplasm (Fig. 1G, H). In addition, combined immunofluorescence and FISH assays confirmed this phenomenon (Fig. 1I). Finally, we compared the expression of LINC00857 in a normal pancreatic ductal epithelial cell line (hTERT-HPNE) and a PC cell line, and the results showed that LINC00857 expression was
significantly increased in the PC cell line (Fig. 1J). Taken together, these results implied that LINC00857 was highly expressed in PC and associated with poor clinical prognosis.

3.2 LINC00857 promotes PC cell migration, invasion and epithelial mesenchymal transformation (EMT).

Considering the clinical association between LINC00857 and PC, we explored the biological significance of LINC00857 during the metastasis of PC cells in vitro. We first generated multiple PC cell lines with either silencing (by shRNAs) or overexpression of LINC00857, and the qRT–PCR results confirmed that LINC00857 expression was significantly decreased in Panc-1 and MIA Paca-2 cells stably expressing the shRNA (Fig. 2A). Additionally, LINC00857 overexpression was observed in PC cells stably expressing the pLVX-LINC00857 construct (Fig. 2B).

Notably, EMT is known as the key factor affecting PC cell metastasis. Next, we used western blotting to explore the effect of LINC00857 on modulating EMT-related molecules, and we found that LINC00857 knockdown with shRNAs inhibited the expression of N-cadherin/Vimentin and elevated the expression of E-cadherin, while forced expression of LINC00857 showed the logical inverse regulatory effects (Fig. 2C). In addition, we used wound healing and Transwell assays to evaluate the migration and invasion abilities of PC cells in different contexts. The wound healing assay showed that LINC00857 knockdown suppressed the migration of Panc-1 and MIA PaCa-2 cells (Fig. 2D), and the difference in the healed area was statistically significant (Fig. 2E), while overexpression of LINC00857 promoted migration (Fig. 2F, G). In addition, the Transwell assays demonstrated that lower LINC00857 expression significantly reduced the migration and invasion abilities of PC cells (Fig. 2H, I), while higher LINC00857 expression enhanced these abilities (Fig. 2J, K). These results revealed that LINC00857 facilitated the migration and invasion of PC cells.

3.3 LINC00857 promotes metastasis by enhancing FOXM1 expression in PC both in vitro and in vivo

Since the above results confirmed the role of LINC00857 in promoting the metastasis of PC cells, we expect to further explore the mechanism. Through website prediction, we found that LINC00857 had a high interaction probability with FOXM1, and the prediction scores were 0.9 and 0.97 with the RF classifier and SVM classifier, respectively (Table S1). Moreover, FOXM1 has been shown to promote the metastasis of PC in previous studies. Hence, we wondered whether LINC00857 could influence PC metastasis by regulating FOXM1. As Fig. 3A shows, FOXM1 expression was detected in Panc-1 and MIA Paca-2 cells with different LINC00857 expression levels. LINC00857 knockdown significantly inhibited FOXM1 protein expression, while overexpression of LINC00857 increased FOXM1 protein expression but not its mRNA level (Fig. S2A).

Then, we wanted to determine whether LINC00857's promotion of PC metastasis was dependent on FOXM1. si-FOXM1 was transfected into LINC00857-overexpressing cells, and EMT-related indicators were detected by western blotting. The results showed that N-cadherin, Vimentin and E-cadherin expression induced by LINC00857 overexpression could be significantly inhibited by si-FOXM1 (Fig. 3B). Moreover, FOXM1 knockdown partially rescued the LINC00857-induced increase in the PC cell wound healing ability
(Fig. 3C, D). Subsequently, changes in PC cell migration and invasion were further examined, and the results suggested that LINC00857 promoted PC cell migration and invasion and that silencing FOXM1 evidently rescued this phenomenon in Panc-1 and MIA Paca-2 cells (Fig. 3E, F).

Next, we analysed the effect of the LINC00857/FOXM1 signalling pathway on tumour metastasis in vivo. Here, a xenograft metastasis model was established by injecting luciferase-expressing cells into BALB/c nude mice via the tail vein. The mice were randomly divided into three groups (A, B, C), and LINC00857-overexpressing KPC cells were subsequently injected into the mice in Groups B and C, while the mice in Group A was injected with control Panc-1 cells. Beginning on the third day after successful injection, the mice in Group C were given the FOXM1 inhibitor thiostrepton (17 mg/kg) by intraperitoneal injection two times a week, while the mice in the other groups were treated with saline (Fig. 3G). After three weeks of observation, small animal imaging technology was used to evaluate metastasis in the mice. By comparing Group A with Group B, we found that LINC00857-overexpressing PC cell injection resulted in higher fluorescence in lung tissues, and this fluorescence was reduced by administration of the FOXM1 inhibitor thiostrepton (Fig. 3H, I). In addition, mice injected with LINC00857-overexpressing cells (Group B) developed more metastatic lung nodules than control mice (Group A). Similarly, we found that the number of pulmonary metastatic nodules was also significantly reduced after thiostrepton treatment in the comparison between Groups B and C (Fig. J, K). Finally, the metastatic lung tissue samples were fixed and sectioned for staining. The HE staining results showed that the visual field area of lung metastasis was most extensive in Group B, followed by Groups A and C. IHC staining also confirmed that the percentage of FOXM1-positive cells in Group B was significantly higher than that in the other groups (Fig. 3L, M). Collectively, these results suggested that overexpression of LINC00857 enhanced the metastasis of PC, and the effect was inhibited by FOXM1 inhibitors both in vitro and in vivo.

3.4 LINC00857 stabilizes FOXM1 via OTUB1-mediated deubiquitination

Given that LINC00857 affected the FOXM1 protein level but not its mRNA level, we hypothesized that LINC00857 regulates FOXM1 through protein degradation. Common protein degradation methods always include the ubiquitin–proteasome pathway and the lysosomal pathway. Next, cycloheximide (CHX, an inhibitor of protein synthesis), MG132 (a proteasome inhibitor), and chloroquine (CQ, a lysosomal inhibitor) were applied in further experiments. We divided the PC cell interventions into three groups: CHX alone, CHX combined with MG132, and CHX combined with CQ. FOXM1 protein levels were analysed at 0, 1, 2 and 4 hours after intervention. The results showed that FOXM1 protein expression after MG132 treatment was higher than that after CQ treatment, which implied that FOXM1 was degraded mainly through the ubiquitin–proteasome pathway (Fig. S2C). Hence, we induced FOXM1 protein degradation using CHX in PC cells with different expression levels, and the results suggested that LINC00857 knockdown accelerated the degradation of the FOXM1 protein (Fig. 4A, B), while LINC00857 overexpression resulted in slower FOXM1 degradation (Fig. 4C, D). We then examined the ubiquitination level of FOXM1 and found after immunoprecipitation of endogenous FOXM1 in Panc-1 and MIA Paca-2 cells, obviously increased ubiquitin signals were detected in cells with stable LINC00857 silencing
compared to the corresponding control cells. Consistent with this finding, FOXM1 ubiquitination was lower in cells overexpressing LINC00857 than in control cells (Fig. 4E).

Next, we explored how LINC00857 inhibits FOXM1 ubiquitination. Accumulating evidence has demonstrated that IncRNAs may function as scaffolds for binding proteins; thus, we hypothesized that LINC00857 recruits a deubiquitinase to bind FOXM1. To test this hypothesis, we performed RNA pull-down assays with PC cells and observed that multiple target proteins were pulled down by LINC00857, including the deubiquitinase OTUB1 (Fig. S3A). OTUB1 has been reported to block FOXM1 ubiquitination; thus, we further verified this finding. First, we found that OTUB1 knockdown reduced the FOXM1 protein level (Fig. 4F) but did not affect its mRNA expression level (Fig. S3B). Other experiments also confirmed that OTUB1 slowed FOXM1 degradation and reduced the FOXM1 ubiquitination level (Fig. S3C, S3D). Additionally, the western bloting results showed that LINC00857 knockdown did not affect the OTUB1 protein level (Fig. S3E), which means that LINC00857 does not regulate FOXM1 by altering OTUB1 expression but instead via another mechanism. Finally, we simultaneously knocked down OTUB1 in LINC00857-overexpressing cells, and the results showed that FOXM1 upregulation induced by LINC00857 was partially inhibited (Fig. 4E), and the decrease in FOXM1 ubiquitination induced by LINC00857 was also reversed by siOTUB1 (Fig. 4F). Collectively, the results suggested that LINC00857 may reduce ubiquitination-mediated degradation by recruiting the deubiquitinase OTUB1.

3.5 LINC00857 serves as a protein scaffold that promotes the interaction between FOXM1 and OTUB1

Accumulating evidence has demonstrated that IncRNAs may function in different ways during cancer development, including as scaffolds for protein interactions. Therefore, we hypothesized that LINC00857 may provide a scaffold for the interaction between OTUB1 and FOXM1, which increases FOXM1 deubiquitination. According to the previous RNA pull-down/mass spectrometry results, OTUB1 can be pulled down by LINC00857 (Fig. S3A). To verify this, we performed independent RNA pull-down assays in both Panc-1 and MIA PaCa-2 cells and found that OTUB1 was successfully pulled down by LINC00857 compared with its antisense RNA as the negative control (Fig. 5A). Furthermore, RIP assays suggested that LINC00857 was enriched in RNA–protein complexes precipitated with anti-OTUB1 antibody in PC cells (Fig. 5B). Combined immunofluorescence and FISH analysis showed that the colocalization of LINC00857 and OTUB1 was mainly in the nucleolus and partly in the cytoplasm (Fig. 5C). Similarly, we found that FOXM1 was pulled down by LINC00857 (Fig. 5D), and qRT–PCR results indicated obvious enrichment of LINC00857 mRNA with an anti-FOXM1 antibody (Fig. 5E). Moreover, we also confirmed the colocalization of LINC00857 and FOXM1 (Fig. 5F).

The above results indicated that LINC00857 can bind separately to FOXM1 and OTUB1. We then set out to demonstrate the effect of this binding on the interaction between FOXM1 and OTUB1. To this end, we first revealed the interaction between OTUB1 and FOXM1. Co-IP assays showed that FOXM1 could be precipitated with OTUB1 and that endogenous OTUB1 could be precipitated with FOXM1 in Panc-1 and MIA Paca-2 cells (Fig. 5G). Moreover, immunofluorescence assays demonstrated that FOXM1 and OTUB1 were colocalized with each other in PC cells (Fig. 5H). As shown in Fig. 5I, we then knocked down
LINC00857, and the results suggested that less FOXM1/OTUB1 protein was precipitated with anti-OTUB1/FOXM1 antibodies from Panc-1 cells in comparison with the corresponding control cells. In contrast, more FOXM1/OTUB1 protein was precipitated with anti-OTUB1/FOXM1 antibodies in cells with stable forced expression of LINC00857 compared to the corresponding control cells (Fig. 5J), and similar results were confirmed in MIA Paca-2 cells (Fig. 5K).

Since we attempted to demonstrate the role of LINC00857 as a scaffold, we were eager to clarify the possible binding site. Then, we applied ChIRP, in which we used 20 biotin-labelled probes for LINC00857 segments to pull down FOXM1 and OTUB1 separately (Fig. 5L). The results showed that compared with the control probe, probes 2 and 7 could pull down FOXM1, while OTUB1 could be pulled down by probes 3, 6 and 10 (Fig. 5M), which means that the binding of LINC00857 to FOXM1 may be localized between nt 422–441 or 1253–1272 and that the binding of LINC00857 to OTUB1 may be localized between nt 51–70, 401–420 or 721–740 (Fig. 5N). Collectively, these results suggested that LINC00857 may function as a scaffold to facilitate interactions between FOXM1 and OTUB1, which could decelerate FOXM1 degradation.

3.6 LINC00857 is transcriptionally regulated by mutant p53

As p53 mutation is one of the predisposing factors for the development of PC, we interestingly observed that the mutant p53 group expressed a higher level of LINC00857 than the wild-type p53 group in the TCGA database (Fig. 6A). Thus, we speculated that LINC00857 could be regulated by mutant p53. Panc-1 and MIA Paca-2, two p53 mutant cell lines, were convenient for further study. We knocked down and overexpressed mutant p53 in these two cell lines and verified the knockdown and overexpression efficiencies; the results showed that mutant p53 expression was successfully changed at the mRNA (Fig. 6B, C) and protein (Fig. 6D) levels. Indeed, depletion of mutant p53 significantly abolished LINC00857 expression (Fig. 6E). In contrast, the expression of LINC00857 was increased with mutant p53 overexpression (Fig. 6F). To confirm the possible binding site, we searched the JASPAR and PROMO databases. Sequence analysis showed that the LINC00857 promoter contains 5 putative binding sites for mutant p53 (Fig. 6G). Based on these binding sites, we designed 5 pairs of primers (P1-P5) and conducted ChIP-qPCR experiments, and the results revealed obvious enrichment of P1 and P5 but not P2-P4 by the anti-p53 antibody (Fig. 6H); this result was also confirmed by DNA gel electrophoresis in PC cells (Fig. 6I). Then, dual-luciferase reporter plasmids containing the wild-type (WT) or mutant (MUT) promoter sequence were designed and transfected into Panc-1 and MIA Paca-2 cells (Fig. 6J). The results showed that overexpression of mutant p53 significantly increased the luciferase activity in the WT2 group, while no obvious changes were observed in the MUT2, WT1 and MUT2 groups (Fig. 6K), which means that mutant p53 binding at LINC00857 promoter P4 promotes transcription. Therefore, these data strongly indicated that LINC00857 was transcriptionally regulated by mutant p53 in PC cells.

3.7 Mutant p53-LINC00857-mediated metastasis of PC was inhibited by atorvastatin

Statins are among the most commonly used lipid-lowering drugs in clinical practice, and recent studies have reported that statins can degrade mutant p53. Here, we applied one of the commonly used drugs in
the clinic, ATOR (Fig. 7A), to treat cells and observed its regulatory effect on mutant p53 and LINC00857. Using a CCK-8 assay, we evaluated the viability of Panc-1, MIAPaCa-2 and BxPC-3 pancreatic cancer cells treated with different doses of ATOR. ATOR significantly reduced the growth of pancreatic cancer cells in a dose-dependent manner, and its half-maximal inhibitory concentrations (IC50) in Panc-1 and MIA PaCa-2 cells were 43.04 µM and 27.63 µM, respectively (Fig. 7B, 7C). Then, ATOR was applied to PC cells, and we found that ATOR significantly downregulated the protein expression of mutant p53 and the mRNA expression of LINC00857 (Fig. 7D, 7E). Previous data suggested that LINC00857-FOXM1 can promote metastasis in PC. Therefore, we further verified whether ATOR can inhibit PC metastasis, and the results indicated that ATOR elevated the expression of E-cadherin and reduced the expression of N-cadherin/Vimentin (Fig. 7F), which seemed to influence the EMT behaviour of PC cells. Moreover, through Transwell assays, we found that ATOR suppressed the migration and invasion of Panc-1 cells in a dose-dependent manner (Fig. 7G, 7H), and a similar effect was confirmed by a Transwell assay in MIA PaCa-2 cells (Fig. 7I, 7J). Taken together, our data suggested that ATOR could inhibit p53/LINC00857 axis mutation-mediated pancreatic cancer cell metastasis.

Discussion

The incidence of PC has increased annually, and although diagnostic methods and treatment strategies have advanced, the prognosis is still poor. In most PC patients, metastasis has already occurred and the opportunity for surgical treatment has been lost at the time of pathological diagnosis [12]. Therefore, exploring the molecular mechanism of PC metastasis provides a strong theoretical basis for its diagnosis and treatment. As the second most common type of mutation in PC, most p53 alterations are missense mutations that result in gain-of-function phenotypes that include increased metastasis. The tumour-promoting effects of mutated p53 can occur in a variety of ways, including the activation of metastasis-related molecules. LncRNA, as a mostly noncoding molecule, also plays an important role in undertaking mutant p53 and downstream effector molecular functions. In this study, by profiling the transcriptome of PC and normal tissues from the TCGA and GTEx databases, we identified LINC00857 as a LncRNA significantly enriched in PC lesions compared to normal tissues, and TCGA database analysis also showed that LINC00857 was significantly associated with PC prognosis. Moreover, by analysing mutation data in TCGA, we found that LINC00857 was significantly overexpressed in the mutant P53 group compared with the wild-type p53 group. This interesting phenomenon led us to focus on the function and mechanism of LINC00857 in the following exploration.

We further indicated that downregulation of LINC00857 impaired the migration, invasion, and EMT ability of PC cells, while its overexpression significantly promoted PC cell metastasis. Therefore, LINC00857 is believed to play a key role in the metastasis of PC. Regarding the mechanism by which LINC00857 regulates pancreatic cancer metastasis, FOXM1 was selected as a potential research object in view of the website prediction and the previous research of our research group. FOXM1 is a transcriptional activator involved in PC cell metastasis. Xie et al. suggested that FOXM1 directly bound to the promoter region of the Cav-1 and uPAR genes and positively transactivated its activity, which promoted PC metastasis [13, 14]. In addition, our previous studies also confirmed the promoting role of FOXM1 in PC metastasis [15].
In this study, we were the first to comprehensively identify the relationship between LINC00857 and FOXM1. First, LINC00857 was shown to coregulate the protein level but not the mRNA expression level of FOXM1. Therefore, we focused on posttranslational modification, and unsurprisingly, LINC00857 knockdown slowed FOXM1 protein degradation via the ubiquitin–proteasome pathway. Finally, the data confirmed that LINC00857 promotes the metastasis of pancreatic cancer partly through a mechanism dependent on FOXM1. To show how LINC00857 affects FOXM1 degradation, an RNA pulldown-MS assay was performed, which showed that the deubiquitinase OTUB1 could be pulled down by LINC00857. Studies have shown that one of the functions of OTUB1 is to prevent the binding of the target protein ubiquitin to its ubiquitin ligase, thus resulting in its deubiquitination. Through a review of the literature, we found that OTUB1 can bind to FOXM1 and reduce the ubiquitination level of FOXM1 [16, 17]. Therefore, it was reasonable to conclude that LINC00857 promoted FOXM1 protein expression by regulating OTUB1. However, the results showed that LINC00857 did not affect OTUB1 expression; thus, we began to investigate their mutual interaction.

Recent studies have highlighted the critical roles that LINC00857 may play in modulating cancer progression by binding to proteins and regulating their processing, stability, localization, and modification. Chen et al. revealed that LINC00857 can bind to the Y-box binding protein 1 (YBX1) protein, prevent it from proteasomal degradation, and increase its nuclear translocation, which regulates cell proliferation, apoptosis, and autophagy[18]. In addition, LINC00857 also recruited YBX1 to form a novel transcription complex and activated the expression of forkhead Box k1 (FOXK1), thus enhancing the expression of vascular endothelial growth Factor C (VEGFC)[19]. In addition, studies have shown that LINC00857 can bind NF-κB to the promoter region of BIRC5, thereby impairing the radiosensitivity of lung cancer [20]. These studies provide evidence that LINC00857 has important protein-binding abilities. Importantly, our study confirmed that LINC00857 binds FOXM1 and OTUB1; moreover, we also found that knocking down LINC00857 reduced the binding strength of FOXM1 and OTUB1, while overexpression increased the binding strength. Therefore, we believe that LINC00857 can form a protein scaffold that enhances the interaction between FOXM1 and OTUB1 and promotes the deubiquitination of FOXM1 by OTUB1. This is similar to the mechanism of action of IncRNAs mentioned earlier by other researchers [21, 22]. Finally, a ChIRP experiment was used to confirm that LINC00857 specifically binds FOXM1 and OTUB1. The result also showed that the biotin-labelled probe fragment could pull down FOXM1 and OTUB1, but this result was only a simple attempt; approaches such as the introduction of point mutations need to be designed to prove the specific binding site between these proteins.

The main novel finding of our study was that LINC00857 overexpression is driven by mutant p53 in PC. It is known that LINC00857 expression in the p53 mutant group is significantly higher than that in the wild-type group, but we would like to further investigate the underlying mechanism of its high expression. To this end, overexpression and knockdown of mutant p53 were performed in PC cell lines, and it was confirmed that knockdown and overexpression of mutant p53 significantly affected the expression of LINC00857 in Panc-1 and MIA Paca-2 cells (p53 mutation). Furthermore, we demonstrated that mutant p53 binds to the promoter region of LINC00857 to promote its transcription. Inhibitors targeting mutant p53 have been studied for many years, but their inhibitory effects need to be further enhanced.
Accumulating evidence has suggested inhibitory effects against mutated p53, including restoring wild-type p53 functions, degrading the mutant p53 protein and blocking the upstream and downstream molecules of mutant p53 [23–26].

Statins, traditionally prescribed for lipid-lowering effects and cardiovascular disease prevention, have been observed for their anticancer properties. Accumulating evidence has shown that statins also have an anticancer effect in PC [27–30]. In the mevalonate pathway, mevalonic acid (MVA) inhibits CHIP ubiquitin ligase-mediated ubiquitination and proteasomal degradation of mutant p53, leading to mutant p53 stabilization [25, 31]. More importantly, one of the effects of statins is to block the production of MVA, thereby resulting in the degradation of mutant p53 and ultimately achieving the effect of cancer suppression[32]. Therefore, ATOR, as one of the statins, was used to verify the inhibition of the mutant p53-LINC00857 axis. We found that ATOR significantly reduced the mutant p53 protein level, the LINC00857 mRNA level, invasion and migration in PC cells. ATOR inhibits the occurrence and development of PC [33–35]. In this study, the internal mechanism of ATOR was further explored, and LINC00857 was taken as its downstream target. Finally, we demonstrated that LINC00857-FOXM1 promotes PC metastasis and that ATOR inhibits this elevation in a model of metastasis established by tumour cell injection into the tail vein. Substantial clinical evidence suggests that statin use is associated with longer survival in patients with PC. However, studies have also indicated that the observed benefit of statins is not mediated through a lipid-lowering pathway. Therefore, further study of the mechanism of action of statins may provide a better theoretical basis for guiding future clinical use.

**Conclusions**

In summary, our study showed that LINC00857 was able to promote PC metastasis both in vitro and in vivo. Mechanistically, we demonstrated that LINC00857 formed a protein scaffold to strengthen the interaction between OTUB1 and FOXM1, which then reduced the FOXM1 ubiquitination level and increased its protein expression level, leading to changes in EMT and alterations in metastasis-related molecules. Moreover, we proved that mutant p53 is the initiating factor of LINC00857 overexpression and that ATOR can inhibit the mutant p53-LINC00857-FXOM1 axis to inhibit PC metastasis (Fig. 8). It is suggested that the mutant p53-LINC00857-FXOM1 axis is a potentially valuable therapeutic target for the inhibition of PC metastasis, and further detailed research, as well as reliable clinical trials, needs to be carried out.

**Abbreviations**

LncRNA
long noncoding RNA
PC
Pancreatic cancer
FOXM1
Forkhead Box M1
OTUB1
OTU domain-containing ubiquitin aldehyde-binding protein 1
ATOR
Atorvastatin
FISH
Fluorescence in situ hybridization
RIP
RNA immunoprecipitation
ChIRP
chromatin isolation by RNA purification
EMT
epithelial mesenchymal transformation

Declarations

Ethics approval and consent to participate

All protocols were approved by the Ethics Committee of the First Affiliated Hospital of Xi’an Jiaotong University, Xi’an, China.

Consent for publication

The authors agrees to publication in the journal.

Availability of data and materials

All data generated or analyzed during this study are available from the corresponding author on reasonable request.

Competing interests

The authors declare no potential conflicts of interest.

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Authors' contributions
WFZ performed most of the experiments, treated data, and wrote the manuscript; WKQ, JTG, MYG, WNZ, and SMZ participated in analyzing the data and organized the Figures; WKQ, CCZ, JJ, HL read and reviewed the manuscript. ZW and QYM provided many ideas and technical guidance. ZW designed the experiment and manuscript. All authors read and approved the final manuscript.

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Not applicable

References


Figures

Figure 1

**LINC00857 is upregulated in PC and associated with poor prognosis.** (A) The volcano plot of differentially expressed lncRNAs between pancreatic cancer and normal tissues from TCGA and GTEx databases. (B) Relative expression of LINC00857 in normal tissues, pancreatic cancer tissues (TCGA and GTEx). (C) Relative expression of LINC00857 in normal tissues, pancreatic cancer tissues from The First
Affiliated Hospital of Xi'an Jiaotong University. (D) Kaplan–Meier plots of pancreatic cancer patients’ overall survival (OS) and disease free survival (DFS) stratified by LINC00857 expression level. (F) Receiver operating characteristic (ROC) curve based on the survival status of patients with stratified by LINC00857 expression level. (G)(H) qRT-PCR detected the subcellular fractions of LINC00857 in Panc-1/MIA PaCa-2 cells (left). The qRT-PCR products were separated by 2% agarose gel electrophoresis; U6 and β-actin were used as markers of the nucleus and cytoplasm, respectively (right). (I) Combined immunofluorescence of the DIPA (blue) and RNA-FISH analysis of LINC00857 (red) in parental Panc-1 and MIA PaCa-2 cells. (J) Relative mRNA expression of LINC00857 in normal pancreas duct cell hTERT-HPNE and different pancreatic cell lines. The scale bar is 20 μm, *P < 0.05 and **P < 0.01 as indicated.
LINC00857 promotes PC cell migration, invasion and epithelial mesenchymal transformation (EMT). (A) Relative expression of LINC00857 in Panc-1 and MIA PaCa-2 cells that stabilized either silenced or overexpressed LINC00857. (B) N-cadherin, E-cadherin and Vimentin protein expression level detected by western blotting in Panc-1 and MIA PaCa-2 cells. (D)(E) Representative images (left) and the healing area (right) per high-power field with LINC00857 knockdown in PC cells. (F)(G) Representative images and
data statistics of wound healing in Panc-1 and MIA PaCa-2 overexpressing LINC00857. (H)(I) Representative images and the number of migration/invasion cells in LINC00857 knockdown PC cells. (J)(K) Representative images and the number of migration/invasion cells in Panc-1 and MIA PaCa-2 overexpressing LINC00857. The scale bar is 0.5 mm, *P < 0.05 and **P < 0.01 as indicated.
LINC00857 promotes metastasis by enhancing FOXM1 expression in PC both in vitro and in vivo. (A) Relative protein expression of FOXM1 in Panc-1 and MIA PaCa-2 cells that stabilized either silenced or overexpressed LINC00857. (B) Cells with overexpressing LINC00857 and transfected with siFOXM1, western blotting analysis of FOXM1, N-cadherin, E-cadherin and vimentin protein levels in different groups. (C)(D) Representative images and the healing area statistical chart with different groups in PC cells. (E)(F) Transwell assay was used to assess the migration and invasion capability. Representative images and the number of cells with different groups in Panc-1 and MIA PaCa-2 cells. (G) The procedure of model establishment of metastatic tumour by tail vein injection. Mice was divided into 3 groups (A, B, C) and treated with different strategies. (H) (I) Tumour formation in the lungs was monitored by bioluminescence imaging and total flux was counted with statistical analysis in different group. (J)(K) The gross morphology and number of tumour nodules was counted in metastatic lungs excised from the mice in different group. (L)(M) Representative images show H&E and FOXM1 IHC staining in metastatic lungs samples of different groups and relative statistical analysis. The scale bar is 0.5 mm, *P <0.05 and **P < 0.01 as indicated.
**Figure 4**

**LINC00857 stabilizes FOXM1 via OTUB1-mediated deubiquitination.**

(A)(B) Control and knockdown LINC00857 cells treated with protein synthesis inhibitor CHX (400 μg/mL) for 0, 1, 2 and 4 h, respectively, and FOXM1 protein expression level detected by western blotting. The line chart indicated relative quantification.

(C)(D) Over-expressed LINC00857 and control Panc-1 and MIA Paca-2 cells treated with CHX for 0, 1, 2 and 4 h, the FOXM1 protein expression level was detected and line chart of degradation.
rate was showed. (E) Western blotting analyses of the ubiquitination of FOXM1 in Panc-1 and MIA PaCa-2 cells that stabilized either silenced LINC00857 or over-expressed LINC00857. (F) Cells were transfected with siNC and siOTUB1 in PC cells, and OTUB1 and FOXM1 protein expressive level detected by western blotting. (G) Over-expressed LINC00857 cells were transfected with siOTUB1 and western blotting showed OTUB1 and FOXM1 protein expressive level of different groups (Vector, LINC00857, LINC00857+ siOTUB1) in Panc-1 and MIA PaCa-2 cells. (H) Western blotting analyses of the ubiquitination of FOXM1 in PC cells with different treated groups.
Figure 5

LINC00857 serves as a protein scaffold that promotes the interaction between FOXM1 and OTUB1. (A) LINC00857 pull-down followed by western blotting validated the interaction with OTUB1 in Panc-1 and MIA PaCa-2 cells. (B) Fold enrichment of LINC00857 expressive level precipitated with antibody against OTUB1 as compared with the IgG control in PC cells. (C) Combined immunofluorescence of DIPA (blue), OTUB1 (green) and RNA-FISH analysis of LINC00857 (red) in Panc-1 and MIA PaCa-2 cells. (D) LINC00857 pull-down followed by western blotting validated the interaction with FOXM1 in Panc-1 and MIA PaCa-2 cells. (E) Fold enrichment of LINC00857 expressive level precipitated with antibody against FOXM1 as compared with the IgG control in PC cells. (F) Combined immunofluorescence of DIPA (blue), FOXM1 (green) and RNA-FISH analysis of LINC00857 (red) in Panc-1 and MIA PaCa-2 cells. (G) Interactions between FOXM1 and OTUB1 was verified via Co-IP in Panc-1 and MIA PaCa-2 cells. (H) Combined immunofluorescence of DIPA (blue), FOXM1 (green) and OTUB1 (red) in PC cells. (I) Schematic diagram of OTUB1’s function on FOXM1. (J)(K) Western blotting analyses of interactions between FOXM1 and OTUB1 was verified via Co-IP in Panc-1 and MIA PaCa-2 cells that stabilized either silenced LINC00857 or over-expressed LINC00857. (L) Flow chart of ChIRP assay. (M)(N) Biotin-labeled LINC00857 segmented probe pull-down followed by western blotting validated the interaction with FOXM1 and OTUB1. The scale bar is 20 μm, *P < 0.05 and **P < 0.01 as indicated.
Figure 6

LINC00857 is transcriptionally regulated by mutant p53. (A) Relative expression of LINC00857 in pancreatic cancer tissues wild p53 group and mutant p53 group (TCGA). (B)(C)(D) Relative expression of TP53 mRNA and mutant p53 protein level in Panc-1 and MIA PaCa-2 cells that either silenced or over-expressed mutant TP53. (E)(F) Relative expression of LINC00857 mRNA in PC cells that either silenced or over-expressed mutant TP53. (G) Schematic images of the potential mutant p53 binding sites in the promoter of LINC00857 are shown. (I) ChIP assay analysis of mutant p53 occupancy at the LINC00857 promoter in Panc-1 and MIA PaCa-2 cells. (J) Schematic images of luciferase reporter plasmids
containing mutant p53 binding sites (WT and MUT) in the LINC00857 promoter region. (K) Luciferase reporter assays of PC cells overexpressing mutant p53 and transfected with reporter plasmids containing WT and MUT LINC00857 promoters. The scale bar is 20 μm, *P < 0.05, **P < 0.01, ns means the difference is not significant.
Mutant p53-LINC00857-mediated metastasis of PC was inhibited by atorvastatin. (A) Chemical structure of atorvastatin. (B) Panc-1 and MIA PaCa-2 cells were treated with ATOR (0-500 μM) for 24 h, and cell viability was determined using the CCK-8 assay. (D)(E) FOXM1 and mutant p53 protein level and LINC00857 mRNA level was detected in different treatment groups. (F) Western blotting analysis of N-cadherin, E-cadherin and vimentin protein levels with different treatments in Panc-1 and MIA PaCa-2 cells. (G)(H)(I)(J) Transwell assay was used to assess the migration and invasion capability. Representative images and the number of cells with different treatments in Panc-1 and MIA PaCa-2 cells. The scale bar is 0.5 mm, *P < 0.05 and **P < 0.01 as indicated.

**Figure 8**

Proposed schematic diagram of mutant p53-LINC00857-FOXM axis in PC metastasis. In general, compared with the absence of mutant p53, mutant p53 binds with the promoter region of LINC00857 and promote its expression. Subsequently, LINC00857 serves as scaffold protein to enhance the interaction between FOXM1 and OTUB1, thereby increased the deubiquitination of FOXM1 and promoted the accumulation of FOXM1, which further promoted EMT and metastasis in PC, and the mutant p53-LINC00857 axis was inhibited by atorvastatin.

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