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The progression of pancreatic cancer cells is promoted by a long non-coding RNA LUCAT1 by activating AKT phosphorylation

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Abstract. – **OBJECTIVE: In many cancers, long non-coding RNAs (lncRNA) are largely involved; they can regulate cell proliferation, migration, and invasion. However, the research of lncRNA regulation on pancreatic ductal adenocarcinoma is vacant. The aim of this article was to lucubrate the specific role of lncRNA LUCAT1 in regulating the progression of pancreatic cancer.**

PATIENTS AND METHODS: Pancreatic cancer and adjacent tissues were collected, and the expression of LUCAT1, one potential involved LucRNA, was measured using real-time qPCR (RT-qPCR). Different pathological types of pancreatic cancer cell lines were cultured, and the expression difference of LncRNA LUCAT1 was detected by RT-qPCR, and two cell lines were selected for downstream experiments. si-RNA was used to knockdown the expression of LUCAT1, comparing the difference in expression of LUCAT1, characterizing cell proliferation by MTT and BrdU staining, detecting apoptosis, and cell cycle changes by flow cytometry. Meanwhile, Western blotting was used for the detection of cyclin expression and thus investigate two important associated signaling pathways. Besides, the expression of signaling pathway was validated by signaling inhibitor.

RESULTS: In comparison to normal cells, LUCAT1 was highly expressed in human pancreatic cancer cell lines (p<0.05). The higher expression of LUCAT1 resulted in enhanced pathogenesis of PDA cells and motivated the development to S phase by regulation of cyclin D1, CDK4. Furthermore, LUCAT1 promoted PDA cells development by inducing AKT's and p38 MAPK's phosphorylation.

CONCLUSIONS: LUCAT1, as the key factor, played a positive role in the proliferation and invasion of pancreatic cells *via* AKT/MAPK signaling.

Key Words:

Pancreatic cancer, Proliferation, Invasion, Long non-coding RNA, LUCAT1, AKT phosphorylation.

Introduction

A large proportion of pancreatic cancer is pancreatic ductal adenocarcinoma (PDAC). This disease features a high mortality, and in most cases, is typically diagnosed as advanced state[1]. The overall five-year survival probability of pancreatic cancer patients is only 7.7%[2]. Traditionally, PDAC can be treated by surgery and chemotherapy, but only 15% to 20% of patients are eligible for radical surgery, most of them lost the best timing because of the distant metastasis during the diagnosis[3,4]. Clinically, the improvement of patients' survival and quality of life is not satisfactory, and the treatment status of PDAC is still worrying.

In humans, nucleotide sequences that contain over 200 nucleotides and produce no proteins are defined as long non-coding RNAs (lncRNA)[5,6]. Many reports have revealed that lncRNA is abnormally expressed in multiple human cancers[7] and widely participate in cancer cells' multiple biological processes, including proliferation, differentiation, and apoptosis[8]. Also, lncRNA regulates the drug resistance of tumors[9]. Yoon et al[10] reported that lncRNA LUCAT1 may have links with the carcinogenesis of esophageal squamous cell carcinoma and LUCAT1 showed a significantly higher expression in ESCC cell lines and cancer tissue. Besides, Zhe et al[11] researched the potential roles of lncRNAs played in osteosarcoma chemoresistance, and their results suggest that the profound regulatory function of LUCAT1 in the methotrexate resistance and that the miR-200c/ABCB1 pathway is involved in the mechanism. However, the connection between lncRNA and pancreatic cancer was still unknown.

In this study, LUCAT1 was selected as the research target for pancreatic cancer tissues. Be-

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sides, pancreatic cancer and adjacent tissues were collected and detected the differential expression of LncRNA LUCAT1 by qRT-PCR. Following that, how LUCAT1 affect pancreatic cancer cells in terms of their biological function and the associated signaling pathways were also verified.

Patients and Methods

Patients and Tissue Sampling

The samples of pancreatic cancer used in this study were all from surgery. From 2016 to 2017, 10 patients underwent radical or partial pancreatectomy at the Department of Gastroenterology, the Affiliated Hospital of Nantong University. All patients did not undergo radiotherapy and chemotherapy and tumor adjuvant therapy before surgery. Following surgical resection, tumor tissues, together with the matched adjacent non-neoplastic tissues, were collected and stored in liquid nitrogen till use. All samples were diagnosed as PC after the postoperative pathological examination.

Cell Culture and RNA Transfection

We purchased four human pancreatic cancer cell lines, namely, BxPC-3, Capan-1, PANC-1 and SW1990 from the American Type Culture Collection, and they were cultured in complete growth medium according to the instruction of the manufacturer. Other supplements included 1% penicillin/streptomycin and 10% fetal bovine serum (FBS). Immortalized human pancreatic ductal epithelial cells (HPDE6) were ordered from Shanghai Institute of Biochemistry and Cell Biology (Shanghai, China). The culture conditions for the above cell lines were set 5% CO_2 and 37°C on a humidity-controlled incubator.

The small interfering RNAs (siRNAs) for down-regulating gene expressions and lentivirus for up-regulating gene expressions were purchased from Shanghai GenePharma (Shanghai, China). For siRNA transfection, the reagent we used was Lipofectamine RNAiMAX Reagent (Life Technologies, Carlsbad, CA, USA) and all steps followed the protocol provided by the manufacturer. After co-culturing cells with small interfering RNA (siRNA) for 48 h, the qRT-PCR assay was done to quantify the transfection efficiency.

RNA Isolation and Quantitative Real-Time PCR

We used the TRIzol reagent (Life Technologies, Carlsbad, CA, USA) to isolate total RNA. For the step of reversed transcription, PrimerScript RT Master (Takara, Kyoto, Japan) was used. Subsequently, quantitative real-time PCR (qRT-PCR) with SYBR-Green PCR Master mix (Roche, Mannheim, Germany) was performed to investigate the gene expression profiles. The assay was conducted using a LightCycler 96 Real-Time PCR instrument (Roche, Mannheim, Germany). To determine the relative values of gene expression level, GAPDH was served as a normalizer and the $2^{-\Delta\Delta CT}$ method was employed. For the statistical analyses of gene expression levels, we performed paired *t*-tests. Table I lists all primers used in this study.

MTT Assays

Small amounts of pancreatic cancer cells were re-seeded into 96-well petri dish plates 48 hours after RNA transfection. The cell density was adjusted to 5 ×10^3/well for consistent measurements, and thus the final volume for each well was 150 µl. After 24, 36, and 48 hours of incubation, respectively, 20 µL 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) solution was carefully added into each well of the plate. After 4 hours of 37°C incubation, the medium was discarded. For the washing step, 150 µl dimethylsulfoxide (DMSO) was added, and the plates were moved to a shaker for shaking 15 minutes. An enzyme labeling analyzer with the

Table I. The primers and siRNAs.

	Forward (Sense)	Reverse (Antisense)
GAPDH	AATGGGCAGCCGTTAGGAAA	AATGGGCAGCCGTTAGGAAA
LUCAT1	GTGTCAAGCTCGGATTGCCT	GAGCCCACACACTCAGGTTC
siR-LUCAT1	CUCAGUGUCACACAUUUCATT	UGAAAUGUGUGACACUGAGTT
p21	TGCCGAAGTCAGTTCCTTGT	GTTCTGACATGGCGCCTCC
CDK4	CCTCTCTAGCTTGCGGCCT	GTTCCTACGGCCCCATACA
cyclinD1	ATCAAGTGTGACCCGGACTG	CTTGGGGTCCATGTTCTGCT
cyclinE2	CGAGCTCCTGAAATCCTCCTG	GGCGAGTCACCATCTCAGCAA

wavelength set to 490 nm was used to measure the optical density (OD). Three biological replicates were included (three times each).

Bromodeoxyuridine Incorporation Assay

Bromodeoxyuridine (BrdU) incorporation assay was also utilized to estimate the cell proliferation level. 10 μM BrdU (Thermo Fisher Scientific, Waltham, MA, USA) was added and, after 48 hours, colchicine (Sigma-Aldrich, Saint Louis, MO, USA) was added to dilute the concentration into 0.1 μM. Two days later, the collected cells were plated on coverslips, which were pre-treated as following: the surface was covered with saline sodium citrate (SSC) buffer solution (2×, Amresco, Framingham, MA, USA) at a temperature of 56°C; in prior to discarding the buffer solution, the coverslips were subjected to 30 minutes of ultraviolet (6 cm) treatment. For the staining step, the coverslips were treated with Giemsa solution (Sigma-Aldrich, St. Louis, MO, USA) for 10 minutes. After microscopic observation of 100 split phases, the cell division index was calculated.

Invasion Assays

Transwell invasion assay was performed as previously with a Matrigel Invasion Chambers (8 μm, BD Bioscience, Franklin Lakes, NJ, USA). All chambers contain 24-well plates, and serum-free medium and 600 μl medium (10% FBS) were added into the upper chambers and lower chambers, respectively. A relatively large number of cells (2×10^5) were input into the upper chamber. The cells in the upper chamber were discarded. 4% paraformaldehyde was used to fix the cells which have invaded the lower surface, and 0.1% crystal violet was added to stain the cells. To count the cells that show ed migration or invasion, 5 fields that were selected randomly were observed using a microscope. Three replicates were included in all assays.

Flow Cytometry Assay

To evaluate PDAC cells' cell cycle and apoptosis, we performed the flow cytometry assay. In order to understand the cell cycle situation, 2×10^5 cells were initially transferred into 6-well petri dish plates. After being subjected to siRNA transfection for 48 h, cells were washed with PBS. The fixation and staining steps were done with overnight ice-cold ethanol (70%) treatment and propidium iodide (PI), respectively. FACSVerse flow cytometer (Becton Dickinson, San Jose, CA, USA) was the instrument we used to check the cell cycle. With regard to apoptosis, similarly, siRNA transfection lasted 48 h, but annexin V-FITC and PI were used for staining, as recommended in the manufacturer's instructions. The same FACSVerse flow cytometer was employed to determine the cell apoptotic rate. All experiments were repeated 3 times.

Western Blot

Ice-temperature PBS was used to wash cells 3 times. For the RIPA lysis buffer (Thermo Fisher Scientific, Waltham, MA, USA), to have a better lysing result, protease and phosphatase inhibitor mixture (Roche, Mannheim, Germany) were added before lysing. We performed total protein extraction as per the manufacturer's instructions. In prior to western-blot experiments, the Bio-Rad assay system (Bio-Rad Laboratories, Hercules, CA, USA) was used to quantify the protein concentration. Electrophoresis (with 10% sodium dodecyl sulfate-polyacrylamide gels) was performed to separate the protein extract (40 μg). Subsequently, polyvinylidene difluoride membranes were used to receive the separated proteins (Merck Millipore, Burlington, MA, USA). The whole blotting procedure was as following: blocking in skim milk (5%) diluted by TBST, 4°C overnight incubation with primary antibodies, washing the membrane 3 times (10 min per time) with TBST, and 1h horseradish peroxidase-linked secondary antibody incubation at 25°C. For blot detection, we used an enhanced chemiluminescence kit (Merck Millipore, Burlington, MA, USA), and the blots were visualized using a Molecular Imager system (Bio-Rad Laboratories, Hercules, CA, USA). GAPDH served as internal control. 3 replicates were included for experiments.

Statistical Analysis

We used GraphPad Prism7.0 or Student's *t*-test through SPSS (13.0; SPSS Inc., Chicago, IL, USA) to perform all statistical analyses. A two-tailed Student's *t*-test was employed to analyze differences between two groups. Multiple comparisons between groups were performed using analysis of variance (ANOVA) followed by Tukey's post hoc test. When $p<0.05$, the difference was considered as statistically significant.

Results

LncRNA LUCAT1 is Highly Expressed in PDAC Tissues and Cell Lines

To understand the function of LUCAT1 in the development of PDAC, we first used RT-PCR to

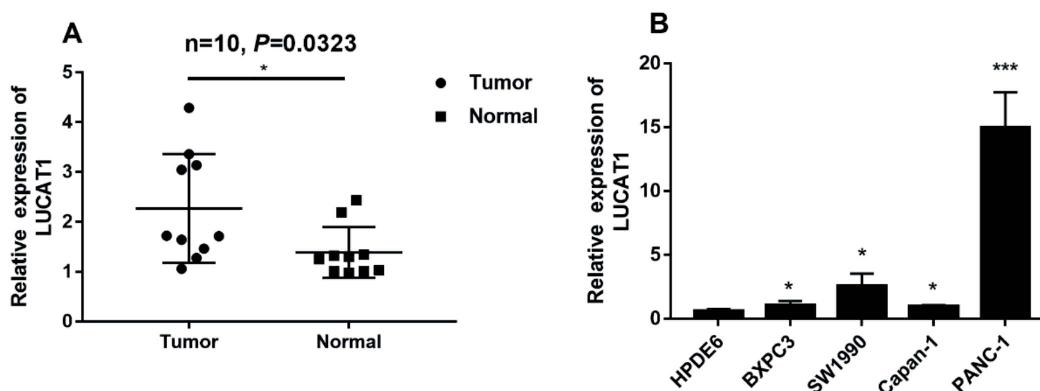

Figure 1. LUCAT1 was highly expressed in pancreatic cancer tissues and cell lines. A, Relative LUCAT1 expression in 10 pairs of PC tissues and normal tissues. B, Relative LUCAT1 expression in human pancreatic cancer cell lines (BxPC-3, Capan-1, PANC-1, and SW1990). The mean value ± SD (n = 3) is shown. *p <0.05, ***p <0.001 *vs.* HPDE6 group by Student's *t*-test.

check the expression of LUCAT1 in 10 pairs of PDAC samples (including adjacent normal tissues). The result showed that LUCAT1 is significantly upregulated in PDAC tissues compared to adjacent normal tissues (p=0.0323), suggesting that LUCAT1 is likely to be a potential oncogene in PDAC (Figure 1A).

We next obtained the expression profiles of LUCAT1 in the human pancreatic cancer cell lines (BxPC-3, Capan-1, PANC-1, and SW1990) and human normal pancreatic ductal epithelial cell line HPDE6. The expression of LUCAT1 in human pancreatic cancer cell lines was significantly higher than that of HPDE6, again suggesting that LUCAT1 has the potential to be considered as an oncogene in PDAC (Figure 1B).

LUCAT1 Promotes Proliferation of Pancreatic Cancer Cells

We next try to understand the possible effects of LUCAT1 on pancreatic cancer cell growth. For down-regulating LUCAT1 expression, siRNA was designed. We found that the expression level of LUCAT1 was significantly down-regulated by siRNA in both PDAC cell lines PANC-1 and SW1990 (p<0.05, Figure 2A). At the same time, lentivirus vector-mediated overexpression enabled us to overexpress LUCAT1 in PDAC cells, as indicated by the LUCAT1 expression data of PANC-1 and SW1990: a significant increase was shown after being transfected with pLV-LUCAT1 in comparison with the empty vector (pLV-NC) control (Figure 2A).

The level of cell proliferation of PANC-1 and SW1990 cells was determined by MTT assay, and LUCAT1 down-regulation conferred a significant inhibition effect on the proliferation of PANC-1 and SW1990 cells. Meanwhile, overexpressing LUCAT1 significantly promoted the proliferation of PANC-1 and SW1990 cells (Figure 2B).

For both PANC-1 and SW1990 cells, BrdU incorporation assay results gave the same tendency that LUCAT1 siRNA transfection resulted in lower BrdU incorporation, suggesting that a lower expression of lncRNA LUCAT1 could down-regulate PDAC cell proliferation. In opposite, higher expression of LUCAT1 showed the positive regulation, that was demonstrated in PANC-1 and SW1990 cells (Figure 2C).

LUCAT1 Facilitates Invasion and Inhibits Aapoptosis of Pancreatic Cancer Cells

The effect of LUCAT1 on PDAC cell invasion was also evaluated. To this end, transwell invasion assays were conducted. Our results showed that down-regulating LUCAT1 significantly limited the PDAC cells from migrating and invading to other tissues. These findings indicated that knockdown LUCAT1 expression could inhibit the invasion of PDAC cells. At the same time, in transwell invasion experiments, over-expressed LUCAT1 was associated with an enhanced invasion of PANC-1 and SW1990 (Figure 3A).

Flow cytometry was used to explore the effect of LUCAT1 on apoptosis of pancreatic cancer cells. It was found that in PANC-1 and SW1990, the portion of apoptosis cells was increased after LUCAT1 was knocked down compared with siR-NC (p<0.001). No significant changes were found in apoptosis after the overexpression of LUCAT1 (Figure 3B). The results indicate that knockdown of LUCAT1 can promote apoptosis of PD cells.

Next, flow cytometry was utilized to explore the function of LUCAT1 on pancreatic cancer cell cycle. LUCAT1 knockdown resulted in significantly increased G0/G1 phase compared to si-NC group and lowered the G2/M phase cell percentage (Figure 4A).

To confirm these results, Western blot and RT-PCR were performed to measure cell cycle checkpoint-associated proteins. CDK4, Cyclin D1 and cyclin E2 showed significantly reduced expression after down-regulating LUCAT1, yet the expression of p21 protein was enhanced. In contrast,

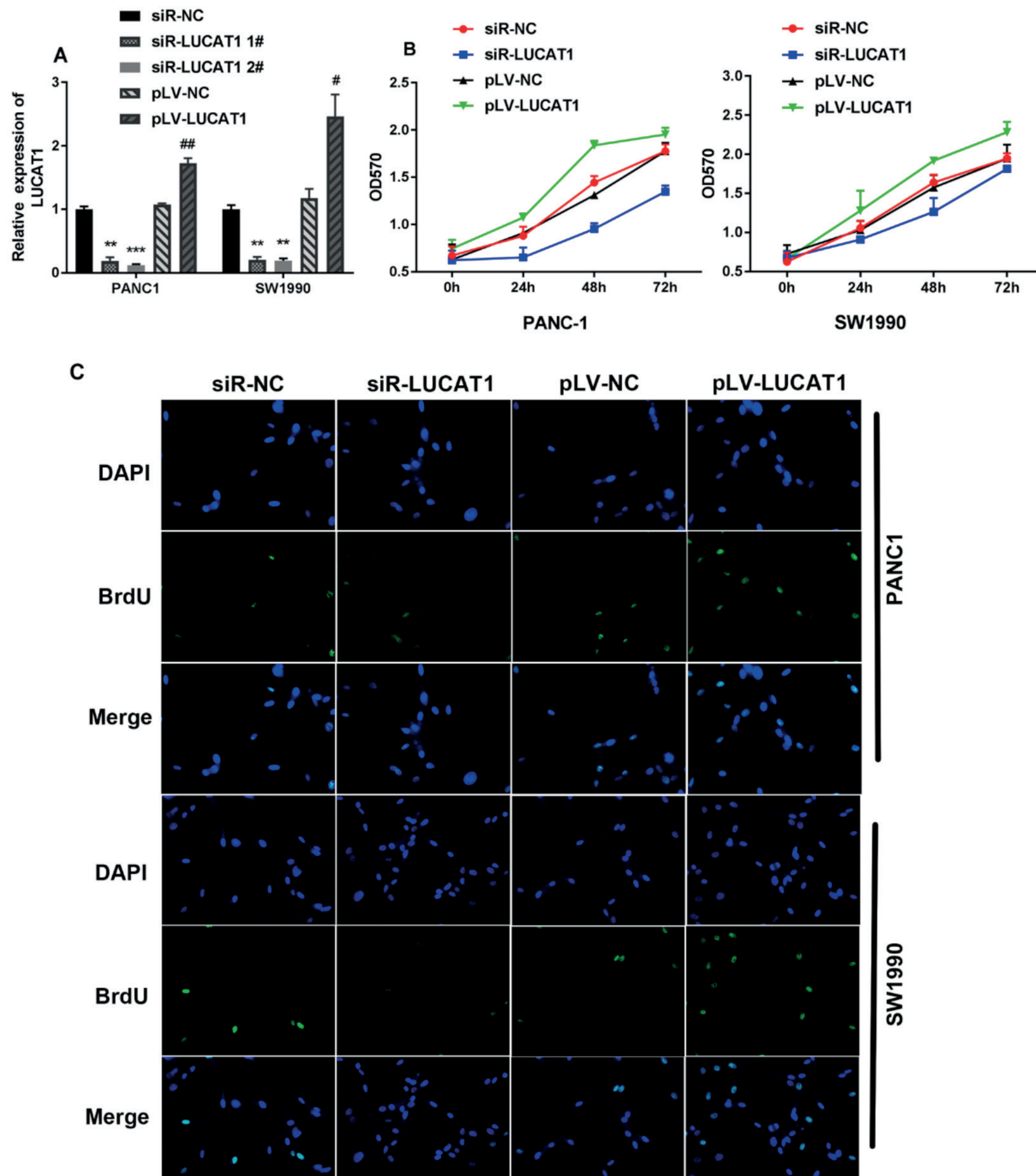

Figure 2. LUCAT1 promotes proliferation of pancreatic cancer cells. **A,** The relative expression level of LUCAT1 in PANC-1 and SW1990 cells. Compared to siR-NC, LUCAT11 significantly reduced LUCAT1, and LUCAT1 expression was significantly increased compared to pLV-NC. **B,** Forty-eight hours after transfection, MTT assays were conducted to determine the proliferation of PANC-1 and SW1990 cells. **C,** The cell proliferation was reduced in the siR-LUCAT1 group in the PANC-1 and SW1990 cell lines by the BrdU incorporation assay (200×), and the proliferation of PLV-LUCAT1 cells was increased as compared with the control pLV-NC group. The mean value ± SD (n = 3) is shown. ***p* <0.01, ****p* <0.001 *vs*. siR-NC group, #*p* <0.05, ##*p* <0.01 vs. pLV-NC group by Student's t- test.

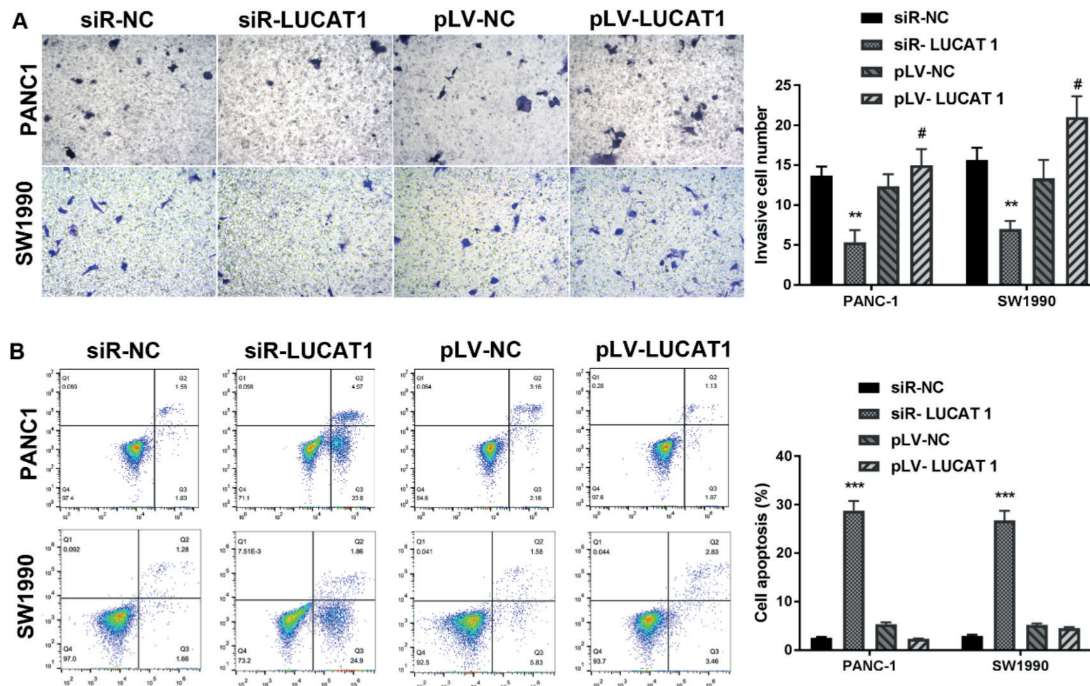

Figure 3. LUCAT1 facilitates invasion and inhibits apoptosis of pancreatic cancer cells. A, Representative images (100×) showing migration or invasion of PDAC cells with LUCAT1 knockdown or overexpression. The histograms stand for the average number of invasive cells observed in one field (5 representative fields in total). **B,** Overexpression and knock down of LUCAT1 in pancreatic cancer cells influenced apoptosis. The mean value ± SD (n = 3) is shown. $^{**}p$ <0.01, $^{***}p$ <0.001 *vs.* siR-NC group, #p <0.05 *vs.* pLV-NC group by Student's *t*-test.

overexpression of LUCAT1 leads to opposite results (Figure 4B-C).

UCAT1 Benefits to AKT and MAPK Signaling Pathways

Molecular mechanisms of the LUCAT1 effect to determine its relevant pathways in pancreatic cancer were also explored in this work. We performed Western blot analysis of key proteins in these pathways. In our results, the expression of p-AKT and p-P38 MAPK was significantly down-regulated after the knockdown of LUCAT1, and the expression of p-AKT and p-P38 MAPK was significantly increased after overexpression, while there was no significant change in AKT and P38 MAPK. Similar results were obtained when LUCAT11 was over-expressed (Figure 5). We initially determined that the effect of LncRNA LUCAT1 on the biological function of pancreatic cancer cells is related to the AKT and MAPK signaling pathways.

AKT pathway is known to have an important role in PDAC. To this, in our LUCAT1 overexpression experiment, we simultaneously added the AKT inhibitor MK-2206 2HCl for inhibiting the AKT pathway. Our results revealed that suppressing the AKT pathway significantly changed the proliferation of PDAC cells and thus limited the effect of LUCAT1 (Figure 6A). At the same time, the Western blotting results indicated that the expression of p-AKT was significantly increased after overexpression of LUCAT1. After the addition of AKT inhibitor, a lower expression of p-AKT was found, while the expression of AKT was not significantly changed (Figure 6B). Taken together, we revealed that the AKT signaling pathway mediates LUCAT1's regulation on the progression of PDAC.

Discussion

A large number of reports have shown that lncRNA is likely to be linked with the occurrence of tumors and may affect their development. Recently, lots of studies revealed that lncRNAs regulated cell proliferation, migration, invasion, associating to pancreatic cancer[12-14]. Pang et al[15] used qRT-PCR to investigate the expression profile of MALAT1, one lncRNA, in pancreatic cancer pa-

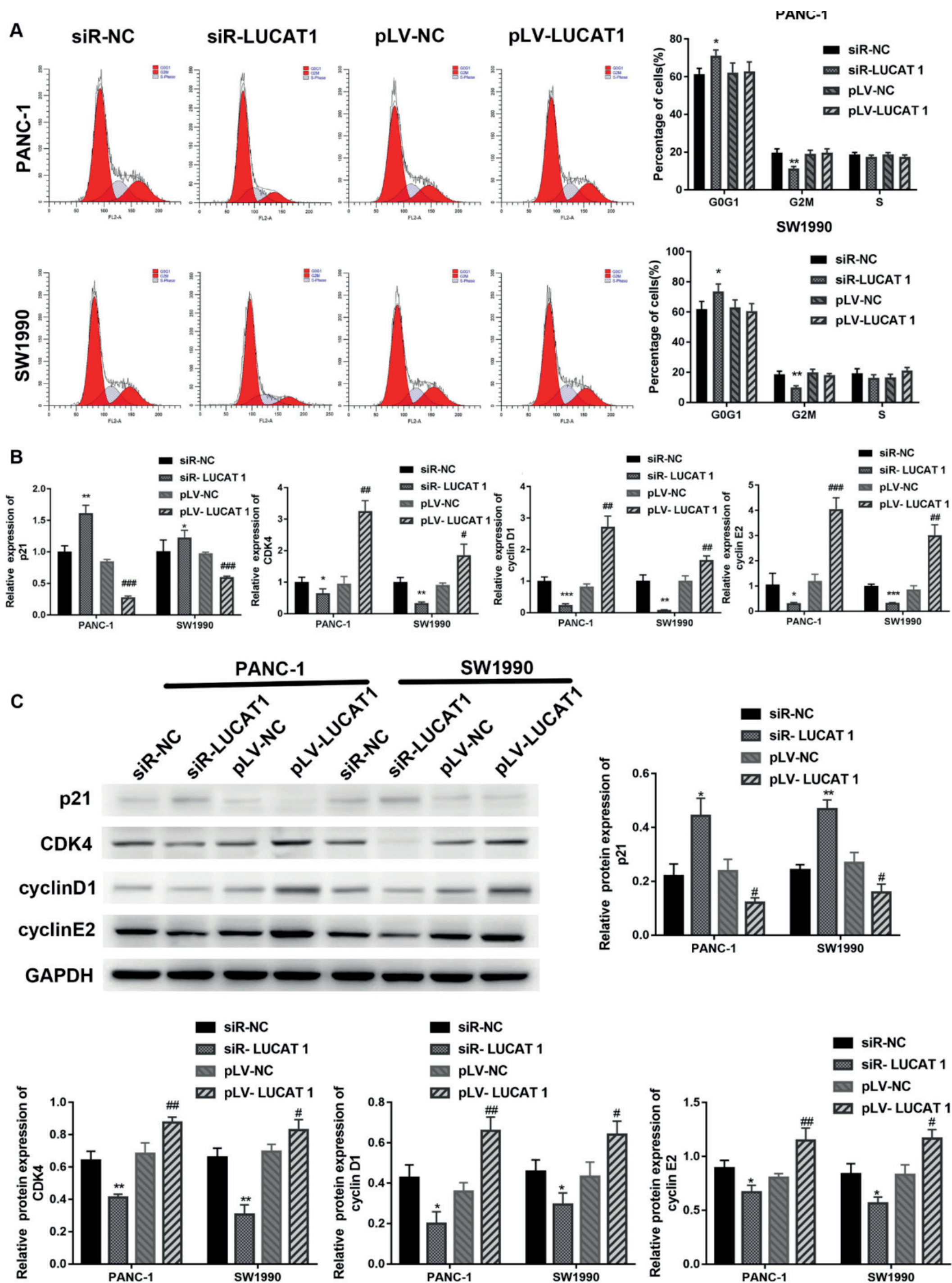

Figure 4. LUCAT1 knockdown leads to pancreatic cancer cells significantly arrested in G0/G1 phase. **A**, Flow cytometric analysis of the cell cycle of knockdown or overexpression of LUCAT1 in PANC-1 and SW1990 cells. **B,** Expression of cyclinD1, p21, CDK4 and cyclinE2 by RT-PCR. **C,** Western blot analysis of cell cycle associated protein expression. The mean value ± SD (n = 3) is shown. **p* <0.05, ***p* <0.01, ****p* <0.001 *vs.* siR-NC group, #*p*<0.05, ##*p* <0.01, ###*p*<0.001 *vs.* pLV-NC group by Student's *t*-test.

tients, and they found distinguished MALAT1 expression between cancer and adjacent non-cancerous tissues.

LUCAT1, also known as SCAL1, was found in the respiratory epithelial cells of smokers and is localized on chromosome 5 and is highly expressed

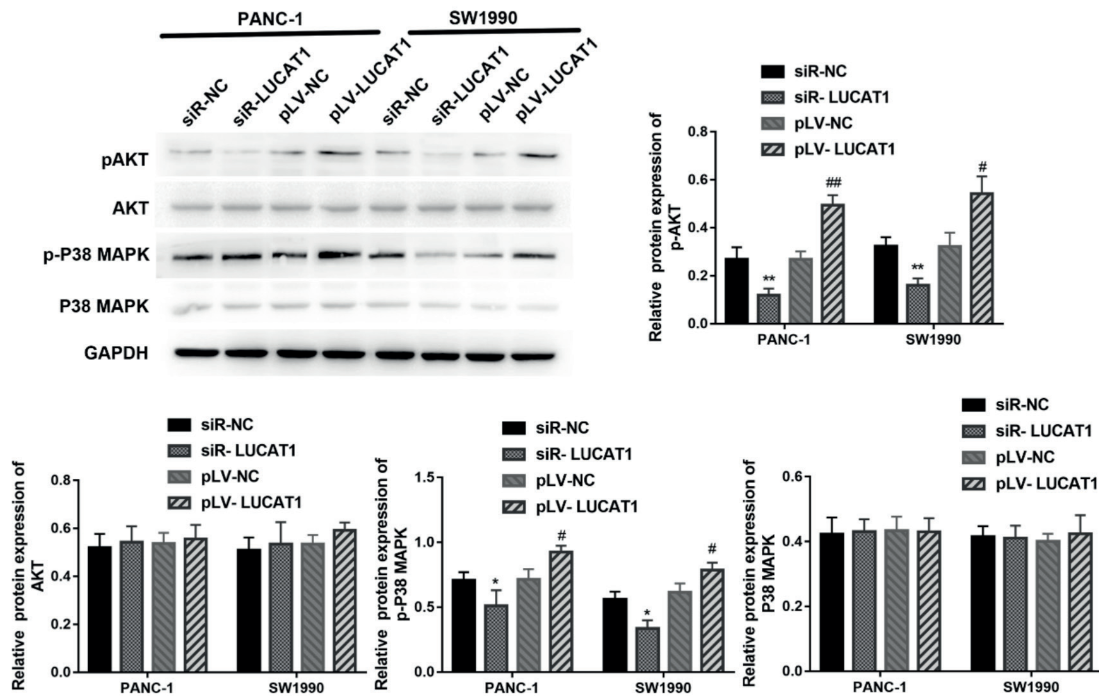

Figure 5. LUCAT1 benefits to AKT and MAPK signaling pathways in pancreatic cancer cells. The Western blot results of related proteins. The mean value ± SD (n = 3) is shown. *p <0.05, **p <0.01 *vs.* siR-NC group, #p<0.05, ##p <0.01 *vs.* pLV-NC group by Student's t-test.

in lung cancer cell lines[16]. Moreover, Han et al[11] investigated LUCAT1's function on osteosarcoma methotrexate (MTX) resistant phenotype, that had a higher expression in MTX-resistant cells. However, the biological regulation of lucat1 in the development of PDAC remains unknown. In the present work, LUCAT1 was overexpressed in 4 PDAC cell lines compared to normal tissues. After that, we silenced lncRNA by si-RNA, that decreased the expression of LUCAT1, leading to the lower viability of PDAC cell lines compared to negative controls, and the result showed significant differences.

The cell cycle regulatory system is a regulatory network that controls the sequence and timing of cell cycle events. A series of biochemical switches trigger cell cycle progression, and there are three major cell cycle regulatory checkpoint transitions throughout cell cycle events[17-19]: The starting point is the G1/S checkpoint, G2/M checkpoint. CyclinD is the initiator of cell cycle and a receptor of growth factor. CyclinD is increased in early G1 expression, cyclinD binds to CDK4 and CDK6 in cells, and cells pass the G1 restriction through cyclinD-CDK4/CDK6 pathway[20-22]. Motokura et al[23] found that its overexpression activates the activity of CDK4 and CDK6 and shortens the G1 phase. CyclinE expression begins in the middle of G1 and peaks at the G1/S junction. When CyclinD binds to CDK4/CDK6, it phosphorylates RB protein, releases transcription factor E2F, induces CyclinE and CDK2 expression, and forms phosphorylation of RB (p-RB) protein. A positive feedback loop that further phosphorylates RB and the cell crosses the G1/S turning point. Western blot results indicate that si-RNA successfully downregulated the expression level of cyclin D1, CDK4 and Cyclin E2 and increased p21 protein expression level, suggesting that si-RNA regulated the cell cycle at G1 phase, inhibiting the development to S/G2.

After that, we used transwell invasion assays to get the evidence that expression of LUCAT1 could influence PDAC invasion. The result suggested that lower expression of LUCAT1 is negatively correlated with cell invasion of PDAC. Conversely, we used pLV-LUCAT1 to achieve the overexpressed LUCAT1, showing the higher expression is positively correlated with cell invasion of PDAC. AKT is a serine/threonine protein kinase, which sometimes is also termed as protein kinase B (PKB). Yang et al[24] have

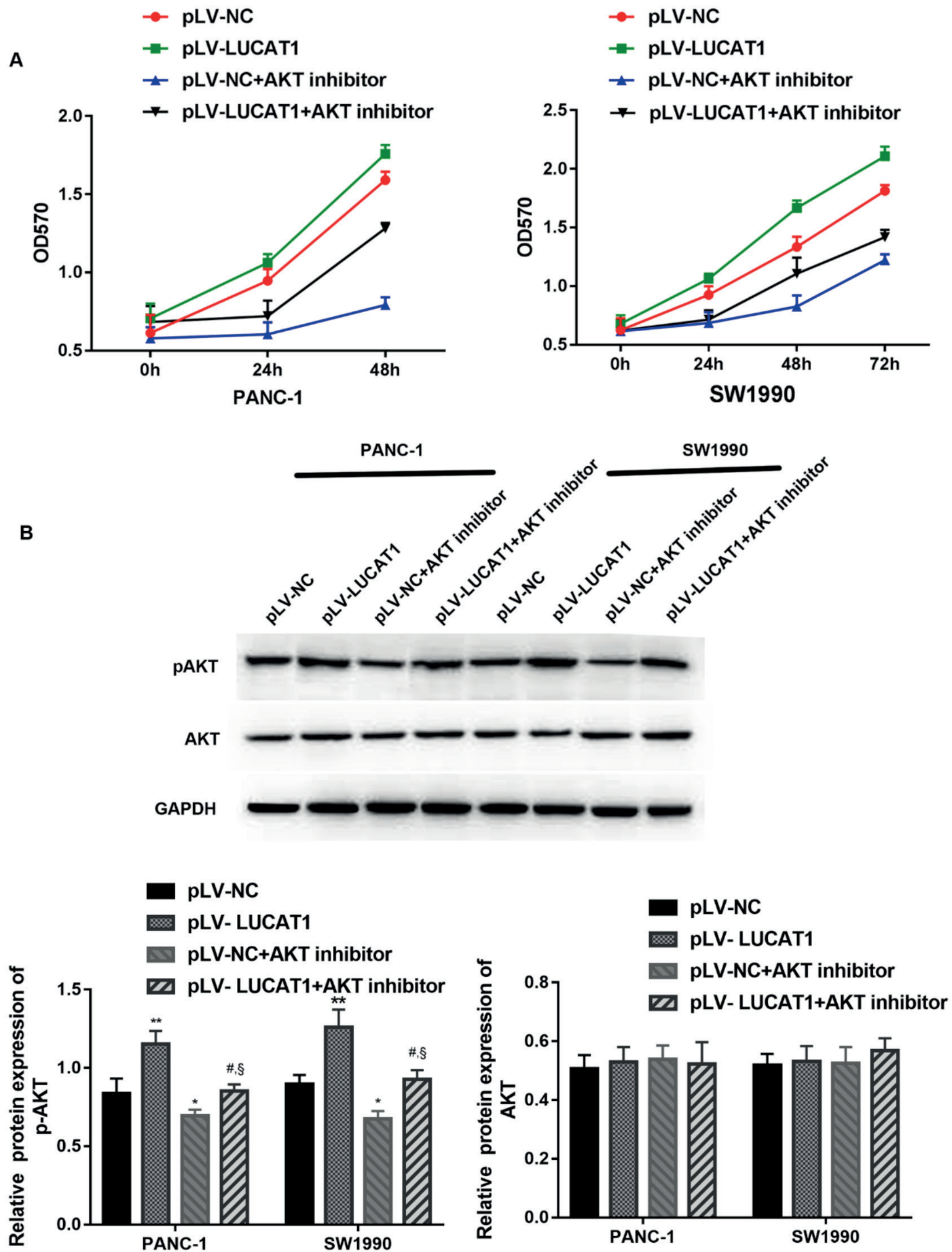

Figure 6. AKT signaling pathway mediated the regulation of LUCAT1 on the progression of pancreatic cancer. **A,** The effect of LUCAT1 overexpression and AKT inhibition on PDAC cell proliferation was analyzed by MTT assay. **B,** Western blotting analysis of the expression of associated pathway proteins in PDAC with LUCAT1 overexpression. *p <0.05, **p <0.01 *vs.* siR-NC group, #p <0.05 vs. pLV-NC group, § p<0.05 vs. pLV-LUCAT1 group by Student's t-test.

shown that the activation of AKT is also one of the most frequent alterations observed in human cancers. Phosphorylation of AKT by PDK1 enhances PI3K, affecting downstream apoptosis-related proteins such as GSK-3β, BAD, FKHR, CaMKII, etc.[25]. The MAPK signaling pathway promotes cell proliferation and regulates gene transcription and is an important factor in many tumorigenesis[26]. The study found that during the apoptosis of pancreatic cancer cells, the three important proteins of the MAPK signaling pathway, ERK/JNK/P38, were phosphorylated[27]. Therefore, it can be determined that the MAPK signaling pathway has a strong link with the apoptosis of pancreatic cancer cells. Thus, LUCAT1 might play an important role in promoting pancreatic cells' progression, where the AKT/MAPK signaling is involved.

Conclusions

Our research suggested that LUCAT1 expression was significantly promoted in pancreatic cancer tissues and was significantly associated with the malignant state and prognosis in pancreatic cancer. LUCAT1 might be a positive regulator in proliferation and invasion in pancreatic cell through AKT signaling, that could be used as a marker for diagnosing PDAC.

Conflict of Interest

The Authors declare that they have no conflict of interests.

Authors' Contributions

All authors have been involved in the research design. WC performed most experiments and data analysis. WC and GZ drafted the initial version of this manuscript. All authors revised and commented on the previous manuscript versions. In prior to submission, all authors approved the final manuscript.

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Conflict of Interest

The Authors declare that they have no conflict of interests.

Ethical Approval

In this study, all human participants related experiments were conducted in agreement with the ethical standards of the Ethics Committee of the Affiliated Hospital of Nantong University as well as the 1964 Helsinki Declaration and its later amendments or comparable ethical standards..

Informed Consent

All individual participants included in the study submitted their informed consent..

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