Original Article

MicroRNA-543 controls pancreatic cancer development by LINC00847-microRNA-543-STK31 axis

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Background: Pancreatic cancer (PC) is one of the most malignant cancers of the gastrointestinal tract. However, the study of targeted therapy research in PC is not very thorough. Therefore, targeted molecular markers are needed to aid in the diagnosis and treatment of PC.

Methods: In our research, we investigated the biological functions and molecular mechanism of microRNA-543 in PC. Western blotting (WB) and quantitative real-time polymerase chain reaction (qRT-PCR) were performed to analyze the transcription and protein expression of microRNA-543, Serine/threonine kinase 31 (STK31), and LINC00847 in BxPC-3 and PANC-1 cells. Subsequently, Cell Counting Kit-8 (CCK-8), Transwell, colony formation, and flow cytometry (FCM) assays were utilized to evaluate cell growth, migration, invasion, and apoptosis. WB and fluorescence in-situ hybridization (FISH) were used to evaluate the epithelial-mesenchymal transition (EMT) process and subcellular localization. RNA immunoprecipitation (RIP), double luciferase reporter, and RNA-pull down assays were performed to determine the targeting relationship between microRNA-543 and STK31 or microRNA-543 and LINC00847.

Results: While microRNA-543 expression was discovered to be low in PC, LINC00847 and STK31 were overexpressed at significant levels. MicroRNA-543 knockdown dramatically increased PC cell growth, invasion, metastasis, and EMT, as well as decreased apoptosis in functional studies. Furthermore, microRNA-543 and STK31 were found to be mutual targets. LINC00847 acted as a molecular sponge for microRNA-543 and a competitive endogenous RNA (ceRNA) for STK31, thereby increasing STK-31 transcription.

Conclusions: Our results suggest that microRNA-543, through the LINC00847/microRNA-543/STK31 axis, plays a role in the development of PC as a tumor suppressor. As a result, microRNA-543 may prove to be an effective diagnostic and therapeutic target for PC.

Keywords: Pancreatic cancer (PC); microRNA-543; LINC00847; biological function; epithelial–mesenchymal transition (EMT)

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Introduction

Pancreatic cancer (PC) is one of the most deadly types of gastrointestinal tract cancer. It is usually recognized at an advanced stage and has a poor prognosis. The 5-year survival rate is less than 10% (1,2). PC is still treated with traditional methods, and only about 20% of patients have an opportunity to undergo surgery, leaving the rest with conservative treatment (3). Recently, targeted therapy and immunotherapy have gradually become the focus of research (4). However, the study of targeted immunity in PC is not very thorough. Therefore, targeted molecular markers are needed for the diagnosis and treatment of PC.

MicroRNA-543 has been reported to control the occurrence and development of various cancers, such as cervical cancer, ovarian cancer, colorectal cancer, and non-small cell lung cancer, among others (5-8). However, less is known about its role in PC. Serine/threonine kinase 31 (STK31) controls tumorigenicity via control of differentiation states, suggesting that STK31 may be controlled by an epigenetic mechanism (9). Recent research reported that STK31 controlled the cell growth and cell cycle of lung cancer cells via the Wnt/beta-catenin pathway (10), maintained the undifferentiated state of colon cancer cells (9), and was a novel biomarker of colorectal cancer (11). Interestingly, there are few studies on the mechanism of STK31 in PC.

Long noncoding RNAs (lncRNAs) are defined as functional gene transcripts that are longer than 200 nucleotides and lack protein-coding capacity (12). A large body of research has found that lncRNAs are engaged in a variety of physiological events, such as the immune system response, inflammatory processes, and the advancement of cancer (13-15). There is increasing evidence that abnormal control of lncRNAs is closely related to the development of cancer, which can be linked to their carcinogenic or anticancer properties (16). LINC00847 has been reported to be involved in apoptotic pathways in kidney cancer (17), contributes to hepatocellular carcinoma development (18), and was shown to be induced by E2F1 to accelerate non-small cell lung cancer development by targeting the miR-147a/IFITm1 axis (19). Despite this, very little is known about its function in PC and the underlying mechanisms that cause the disease.

In this study, bioinformatics was utilized with experimental testing, which found that the abnormal transcription of microRNA-543 may affect the development of PC. As a result of our research, we observed that the tumor promoter LINC00847 has therapeutic significance since it sponges microRNA-543 to target the *STK31* gene. Therefore, the LINC00847/microRNA-543/STK31 signaling axis may be a promising therapeutic target for PC treatment and a novel marker for the diagnosis of PC. We present the following article in accordance with the MDAR reporting checklist (available at https://jgo.amegroups.com/article/view/10.21037/jgo-22-1017/rc).

Highlight box

Key findings

- microRNA-543, through the LINC00847/microRNA-543/STK31 axis, plays a role in the development of PC as a tumor suppressor.

What is known and what is new?

- MicroRNA-543 has been reported to control the occurrence and development of various cancers, such as cervical cancer, ovarian cancer, colorectal cancer, and non-small cell lung cancer, among others
- The role of miR-543 in pancreatic cancer is less known. Then we research the functions of miR-543 by ceRNA network in Pancreatic cancer cells and tissues.

What is the implication, and what should change now?

- How miR-543 plays a role in pancreatic cancer deserves our study, and it may become a target for diagnosis and prognosis of pancreatic cancer.

Methods

Patient tissue samples and ethics statement

Human PC tissues and adjacent tissues were obtained from patients admitted to China-Japan Union Hospital of Jilin University from March 2020 to January 2022. All tissue specimens were stored at –80 ℃ until use. The study was conducted according to the Helsinki Declaration (as revised in 2013) and approval was obtained from the Ethics Committee of China-Japan Union Hospital of Jilin University (No. 20221014001). Before the beginning of the study, each patient signed written informed consent.

Transfection and cell culture

The human PC cell lines BxPC-3, SW1990, and PANC-1 were acquired from Cell Resource Center, Institute of Basic Medical Sciences, Chinese Academy of Medical Sciences (Beijing, China). The human PC cell line CFPAC-1 was

Table 1 The primer sequences in the research

Target gene	Forward/ reverse	Primer sequences
β-Actin	Forward	5'-CATGTACGTTGCTATCCAGGC-3'
	Reverse	5'-CTCCTTAATGTCACGCACGAT-3'
U6	Forward	5'-CTCGCTTCGGCAGCACA-3'
	Reverse	5'-AACGCTTCACGAATTTGCGT-3'
microRNA-543	Forward	5'-AAACATTCGCGGTGCGCGTAC-3'
	Reverse	5'-GCAGGGTCCGAGGTATTC-3'
STK31	Forward	5'-GTCAAGTGTCTGCAAAGAGCTGG-3'
	Reverse	5'-CTGTGGCAATGAGCCTTTCCTC-3'
LINC00847	Forward	5'-CGGCTGGAGCGAAGAGT-3'
	Reverse	5'-GAGAACCGCAGAAGAACG-3' ''

purchased from Kunming Cell Bank, Chinese Academy of Sciences (Kunming, China). The human normal pancreatic cell line H6C7 was purchased from Shanghai Yaji Biological. BxPC-3 cells were cultured in RPMI 1640 medium (Gibco, USA). H6C7, CFPAC-1, and PANC-1 cells were cultured in DMEM medium (Gibco, USA). SW1990 cells were cultured in L-15 Leibovitz Medium. All cell mediums were supplemented with 10% fetal bovine serum (FBS) (Gibco, USA) at 37 ℃ in a humidified atmosphere containing 5% CO_2.

Vectors encoding STK31 (oe-STK31), miRNA inhibitor (microRNA-543 inhibitor), negative control (NC vector), control mimic, miRNA mimic (microRNA-543 mimic), control inhibitor, LINC00847 overexpression (oe-LINC00847), and the overexpression control (vector-NC) lentiviruses for LINC00847 silencing (si-LINC00847) and the silencing control (si-NC) were purchased from Ribobio (Guangzhou, China). The manufacturer's procedure was followed for transfection and infection of cells.

Quantitative real-time polymerase chain reaction (qRT-PCR)

Total RNA was extracted from cells using TRIzol (10296010, Invitrogen, USA) reagent. Reverse transcriptions were performed using the PrimeScript RT Master Mix (code No. RR036A, Takara, China) with random primers for the mRNAs and lncRNAs. Reverse transcriptions were performed using the Mir-X miRNA First-Strand Synthesis Kit (code No. 638315, Takara, China) with specific stem-loop primers for microRNA-543. Subsequently, qRT-PCR reaction was performed using TB Green Premix Ex Taq II (code No. RR820Q/A/B, Takara, China) and Mir-X miRNA qRT-PCR TB Green® Kit (code No. 638314, Takara, China) with an ABI Prism 7500 sequence detection system (Prism® 7500, Applied Biosystems, USA). β-actin and U6 were used as internal controls. The transcription of microRNA-543, STK31, and LINC00847 was quantified by the $2^{-\Delta\Delta Ct}$ method. The primers used in the study are shown in *Table 1*.

Western blotting (WB)

The cell samples were lysed using RIPA buffer (purchased from Beyotime, P0013B, China), which was mixed with a phosphatase inhibitor cocktail. Sodium Dodecyl Sulfate PolyAcrylamide Gel Electrophoresis (SDS-PAGE) was utilized to isolate the proteins, which were then deposited to polyvinylidene fluoride (PVDF) membranes for further examination and investigation. After blocking with skimmed milk (5%), primary antibodies directed against specific proteins of interest were incubated over PVDF membranes for a short period of time before being utilized. Antibodies were acquired from Abcam in Cambridge, MA (USA) and used at a 1:1,000 dilution for STK31 (ab155172), Slug (ab27568), Snail (ab216347), β-actin (ab8226), and TWIST (ab50887, 0.2–2 μg/mL).

N-cadherin (13116), E-cadherin (14472), and Vimentin (46173) antibodies were bought from Cell Signaling Technology (CST, Danvers, MA, USA) and used at a 1:1,000 dilution. A secondary antibody comprising horseradish peroxidase (HRP, ab205719) was then administered after the sample was washed several times. Lastly, the protein bands were observed using the EasyBlot ECL kit (Sangon, China).

Cell growth assay

Stably transfected PC cells were cultured in 96-well plates (1×10^4 cells per well). Cell viability was assessed using the CCK-8 (C0037, Beyotime, China) assay. The absorbance at 450 nm was then measured with a microplate reader. Growth in the number of novel PC cells was also determined by performing a colony formation experiment (1×10^4 cells per well of 6-well plates).

Transwell assay

For the purpose of monitoring invasion and metastasis,

Transwell migration chambers were utilized (24-well chambers, 354572 Corning, USA). Matrigel (356234 BD, Solarbio, China) was applied to the container inserts prior to cell invasion trials. In both tests, the upper chamber of each well was seeded with 2×10^5 cells in 300 µL of serum-free media. A mixture of 600–800 µL DMEM (E600003, Sangon Biotech) supplemented with 10% FBS (E510008, Sangon Biotech) was used to fill the lower chambers of the Transwell. Subsequently, the cells were incubated for 24 hours. The top chambers were then rinsed with phosphate-buffered saline (PBS, E607008, Sangon Biotech), fixed with 4% paraformaldehyde (PFA, E672002, Sangon Biotech) for 30–60 minutes, and dyed with 1% crystal violet (A600331, Sangon Biotech) to obtain the desired appearance. Photomicrographs of the cell layers were taken with an Olympus BX51 microscope.

Flow cytometry (FCM) analysis

PC cells were digested with trypsin (A003702, Sangon Biotech) without EDTA and washed twice with cold PBS and collected by centrifugation. Then, 5 µL Annexin V-FITC (E606336, Sangon Biotech) and propidium iodide (E606336, Sangon Biotech) were added to the cells (1×10^6 cells/mL) and mixed well, then placed at room temperature without light for 15 minutes. After incubation, 200 µL of 1× binding buffer (E606336, Sangon Biotech) was added, then FCM was utilized to detect apoptosis within 1 hour.

Dual-luciferase reporter gene assay

We generated and cloned a sequence including the putative binding site of LINC00847 and the 3′UTR of STK31 as well as the corresponding mutant sequence (GenePharma, Shanghai, China) into the pGL3 luciferase reporter vector. Lipofectamine 2000 (11668019, Invitrogen, USA) was utilized for all co-transfection assays. The co-transfection of BxPC-3 and PANC-1 cells with transcription plasmids and either a microRNA-543 mimic or a control mimic was carried out. At 24 hours after transfection, evaluation of relative luciferase activity was performed using the Nano-Glo® Dual-Luciferase Reporter kit (E1910, Promega, USA) in accordance with the manufacturer's instructions.

RNA immunoprecipitation (RIP) assay

The RIP assay was carried out in accordance with the manufacturer's instructions using the Magna RIP assay kit, which was purchased from Applied Biosystems (17-704, Sigma-Aldrich). RIP lysis buffer was applied to BxPC-3 and PANC-1 cells prior to the addition of magnetic beads containing control IgG antibodies (17-704, Sigma-Aldrich). Next, RT-qPCR was performed to identify the most abundant RNA fragments.

RNA pull-down assay

GenePharma (Shanghai, China) provided the biotinylated microRNA-543, and mutant and NC were utilized as controls. After the oligonucleotides were synthesized, the lysates from BxPC-3 and PANC-1 cells were treated with M-280 streptavidin magnetic beads (11206D, Invitrogen, USA) for 30 minutes. Subsequently, we extracted the RNA bound to STK31/LINC00847 and performed quantitative real-time PCR to determine its concentration.

RNA fluorescence in-situ hybridization (FISH)

FISH was carried out using the RiboTM FISH kit (Ribobio, China) in reference to previous research steps (20). The probes of LINC00847 were synthesized and modified by RiboBio (Guangzhou, China), and cellular DNA was stained with 4',6-diamidino-2-phenylindole (DAPI) and photographed by confocal microscopy.

Bioinformatics analysis

MicroRNA-543 was found to have abnormal transcription and was associated with poor prognosis in 160 PC patients from the The Cancer Genome Atlas (TCGA) database (21). The StarBase 3.0 (http://starbase.sysu.edu.cn) database and Kaplan-Meier plotter (http://kmplot.com/analysis/index.php?p=service) also showed the same results. Furthermore, RNA22 (https://cm.jefferson.edu/rna22/) and microRNA.org (http://www.microrna.org/) predicted that microRNA-543 interacted with STK31, and previous research also reported that microRNA-543 plays a role in PC by targeting STK31 (22). Then, we selected STK31 as a target gene of microRNA-543 for further research. The probable target lncRNAs of microRNA-543 were identified using Venn diagrams (http://bioinformatics.psb.ugent.be/webtools/Venn/) and the Starbase3.0 and LncBase (http://carolina.imis.athena-innovation.gr) databases. The transcription of lncRNAs was evaluated by GEPIA2 (http://gepia2.cancer-pku.cn/#analysis).

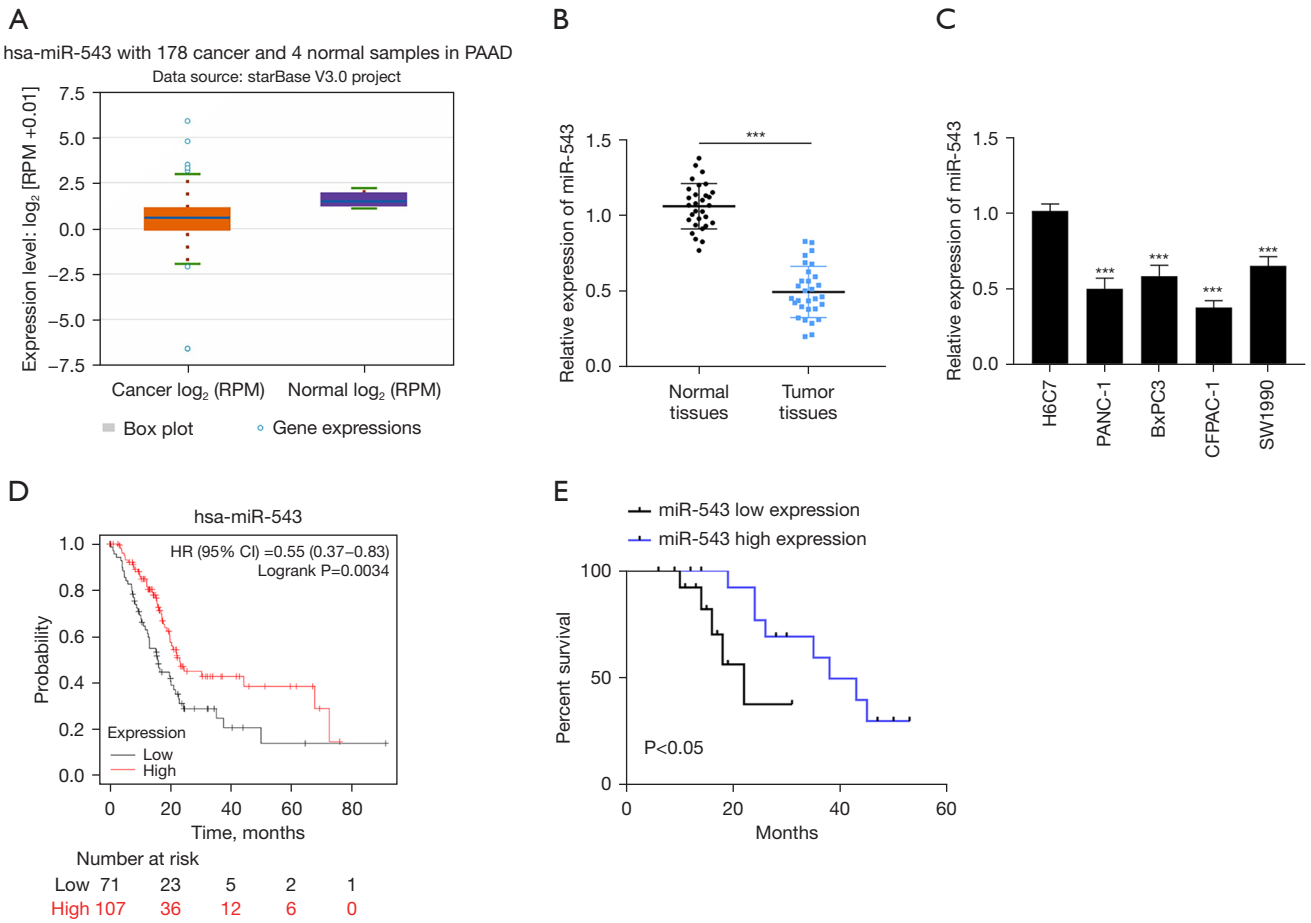

Figure 1 MicroRNA-543 is significantly downregulated in PC and is associated with poor prognosis. (A,B) The expression and survival of microRNA-543 in PC as determined by StarBase and the Kaplan-Meier plotter. (C,D) qRT-PCR analysis of microRNA-543 transcription in PC tissues and cells. (E) Kaplan-Meier survival curves of PC patients with low and high microRNA-543 expression. ***, P<0.001. PAAD, pancreatic adenocarcinoma; PRM, reads per million mapped reads; PC, pancreatic cancer; qRT-PCR, quantitative real-time polymerase chain reaction.

Statistical analysis

The data (mean ± SD) were evaluated by SPSS 22.0 and GraphPad Prism 9. Student's *t*-tests were employed to establish whether or not the data was statistically significant. Kaplan-Meier survival curves were utilized to examine the link between microRNA-543 level and PC patient survival. The Kaplan-Meier survival curves were used to analyze the survival distributions, and the significance of the differences between 2 groups was determined using the log-rank test for the microRNA-543 levels in PC tissue samples. The corresponding graphs were drawn with GraphPad Prism 9, and *&$^{\#}$, P<0.05, **&$^{\#\#}$, P<0.01, or ***&$^{\#\#\#}$, P<0.001 were considered statistically significant.

Results

MicroRNA-543 is downregulated in PC tissues and cells and is associated with poor prognosis

MicroRNA-543 was found to have low expression in 160 PC patients using TCGA database (21). Using the StarBase and Kaplan-Meier plotter databases, we also found that microRNA-543 was downregulated in PC tissues and was associated with poor survival (*Figure 1A,1B*). Furthermore, microRNA-543 was obviously downregulated in PC tissues and cells compared with normal tissues and H6C7 cells (*Figure 1C,1D*, P<0.001). We also collected the clinical data of PC patients and it was found that the expression level of microRNA-543 was significantly correlated with T stage (P<0.05, *Table 2*).

Table 2 The association between microRNA-543 expression and clinicopathological features of pancreatic cancer patients

Characteristics	Case	microRNA-543 expression		P value
		Low (n=20)	High (n=10)	
Age at surgery (years)				0.7888
<60	11	7	4	
≥60	19	13	6	
Gender				0.1927
Male	17	13	4	
Female	13	7	6	
Tumor size (cm)				0.4386
≥3	15	11	4	
<3	15	9	6	
T grade				0.0371*
T1+T2	13	6	7	
T3+T4	17	14	3	
Lymph node invasion				0.7842
Negative (N0)	20	13	7	
Positive (N1-N3)	10	7	3	
TNM stage				0.5921
I-II	19	12	7	
III-IV	11	8	3	
Histological grade				0.0701
Low	16	13	3	
Middle-high	14	7	7	

*, P<0.05. TNM, tumor node metastasis.

Additionally, the Kaplan-Meier method was used to generate overall survival (OS) curves, and survival information of previously registered patients was gathered. Patients who had low levels of microRNA-543 expression in PC tissues had a significantly shorter OS rate (P<0.05, *Figure 1E*). These data indicated that microRNA-543 may be a novel marker in the development of PC.

Knockdown of microRNA-543 promotes the development and epithelial-mesenchymal transition of PC in vitro

In order to study the molecular function of microRNA-543 in PC cells, BxPC-3 and PANC-1 cells were selected to construct stable cell lines to evaluate how microRNA-543 affects the development of PC. As shown in *Figure 2A*, the stable transfection of cell lines was confirmed compared with the control group. CCK-8 and colony formation assays were performed to investigate the growth of PC cells. The results revealed that microRNA-543 silencing promoted the growth of PC cells, while microRNA-543 overexpression suppressed the growth of PC cells compared with controls (*Figure 2B,2C*). In addition, the Transwell assay was performed to evaluate the impact of microRNA-543 expression on the invasion and metastasis of PC cells. From the results, we found that microRNA-543 knockdown substantially enhanced the invasion and migration of PC cells compared with the controls. On the other hand, overexpression of microRNA-543 slowed cell invasion and migration dramatically (*Figure 2D,2E*). Furthermore, the FCM assay was performed to analyze the role of microRNA-543 in the apoptosis PC cells. We found that microRNA-543 silencing substantially inhibited cell apoptosis compared with controls, whereas overexpression of microRNA-543 substantially enhanced cell apoptosis (*Figure 2F*). Finally, EMT marker expression, a key biological process of tumor cell migration and invasion (23), was detected by WB. Interestingly, the results showed that knockdown of microRNA-543 enhanced N-cadherin, Vimentin, Slug, Snail1, and TWIST protein expression and inhibited E-cadherin protein expression in BxPC-3 and PANC-1 cells, indicating a shift from an epithelial to mesenchymal phenotype. When microRNA-543 was increased, PC cells were unable to acquire the EMT phenotype (*Figure 2G*).

MicroRNA-543 controls PC development by targeting STK31

To further explore the function of microRNA-543, we used published bioinformatics articles and online software prediction and found that microRNA-543 acts by targeting STK31 (22). In our study, we further verified the relationship between microRNA-543 and STK31. As shown in *Figure 3A*, we found that STK31 is highly expressed in PC tissues compared with normal tissues. Using qRT-PCR and WB assays, we also found that STK31 expression was significantly higher in BxPC-3 and PANC-1 cells than in H6C7 cells (*Figure 3B,3C*). Then, we transfected microRNA-543 inhibitor and microRNA-543 mimics into BxPC-3 and PANC-1 cell lines and found that the transcription of STK31 was decreased when microRNA-543

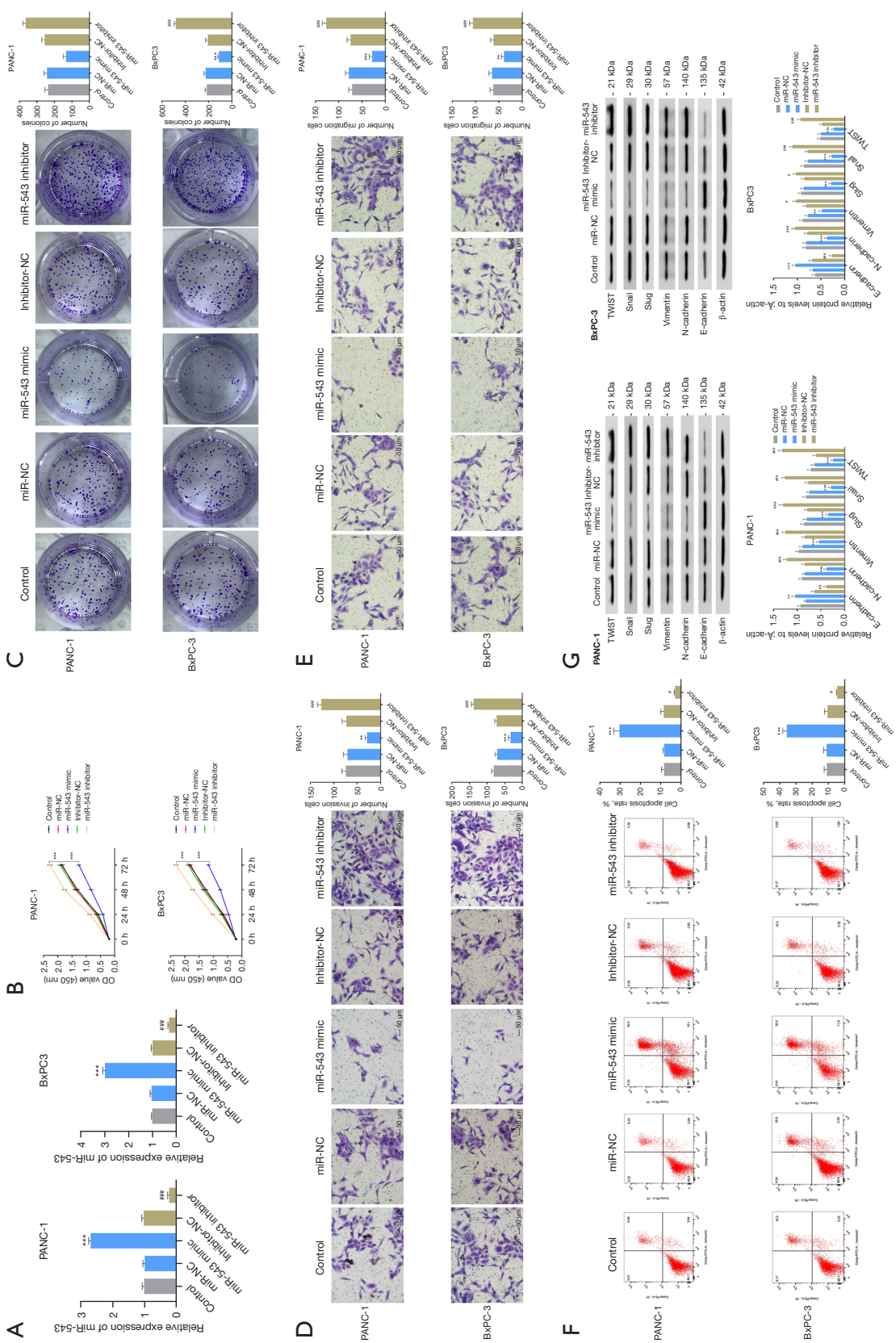

Figure 2 Knockdown of microRNA-543 promoted PC cell growth and EMT in vitro. (A) Stable transfection of PC cell lines was confirmed by the qRT-PCR assay. (B,C) Cell viability and growth of PC cells were evaluated by the CCK-8 and colony formation assays (dyed with crystal violet and taken pictures with Digital Camera). (D,E) The migration and invasion abilities of PC cell lines were assessed by the Transwell assay (dyed with crystal violet, 50 μm). (F) The apoptosis of PC cells was assessed by the FCM assay. (G) The protein expression levels of N-cadherin, E-cadherin, Vimentin, Slug, Snail, and TWIST were detected in the overexpression and silencing microRNA-543 groups by WB. **, P<0.01, ***, P<0.001, #, P<0.05, ##, P<0.01, ###, P<0.001. EMT, epithelial–mesenchymal transition; PC, pancreatic cancer; qRT-PCR, quantitative real-time polymerase chain reaction; FCM, flow cytometry; TWIST, twist family bHLH transcription factor 1; WB, western blotting.

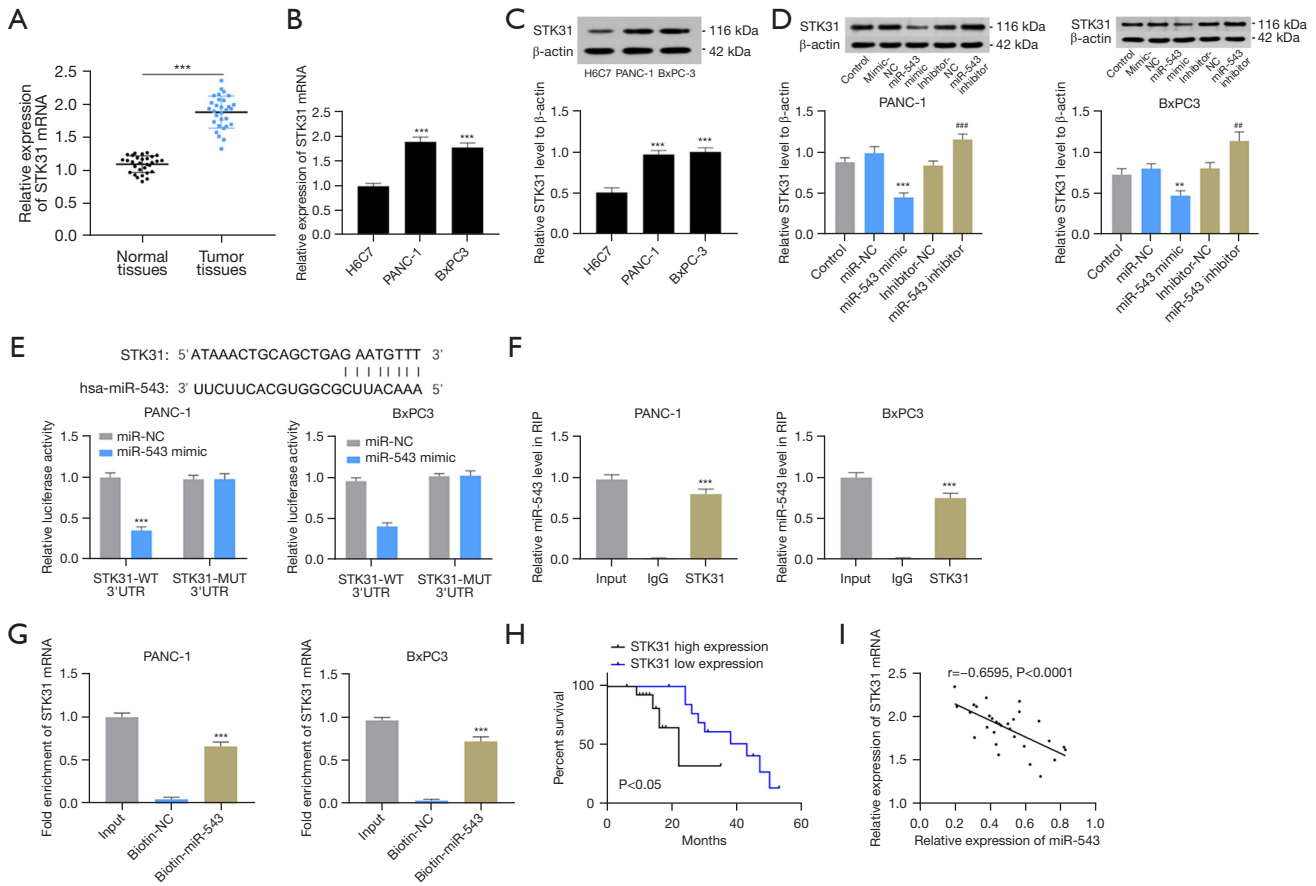

Figure 3 STK31 interacts with microRNA-543. (A,B) The mRNA expression of STK31 were detected by qRT-PCR in PC tissues and cells. (C) The protein expression of STK31 were detected by WB in PC cells. (D) Using WB analysis, it was found that the microRNA-543 mimics or inhibitors transfected into BxPC-3 and PANC-1 cells impaired the elevation of STK31 protein expression. (E) Structural and functional interactions between STK31 and microRNA-543 were tested using luciferases. (F) RIP assay showing the fold enrichment of STK31 and microRNA-543 using IgG antibodies in PANC-1 and BxPC-3 cells. (G) The levels of STK31 and microRNA-543 enrichment in BxPC-3 and PANC-1 cells were evaluated by the RNA pull down assay. (H) Kaplan-Meier survival curves of PC patients with low and high STK31 expression. (I) Pearson correlation analysis of the correlation between STK31 and microRNA-543 expression based on PC tissues. **, P<0.01, ***, P<0.001, ##, P<0.01, ###, P<0.001. qRT-PCR, quantitative real-time polymerase chain reaction; WB, western blotting; PC, pancreatic cancer; RIP, RNA immunoprecipitation.

was overexpressed, while the transcription of STK31 was increased when microRNA-543 was knocked down (*Figure 3D*). The predicted binding site for microRNA-543 and STK31 is shown in *Figure 3E*. The interaction was verified by a luciferase reporter assay. A decrease in luciferase activity was seen in BxPC-3 and PANC-1 cells expressing the wild-type (WT) STK31 reporter, whereas the luciferase activity in cells expressing the mutant (MUT) STK31 reporter was unaffected (*Figure 3E*). The direct interaction between STK31 and microRNA-543 was further confirmed by RIP and RNA-pull down assays, which demonstrated that STK31 directly binds to microRNA-543 (*Figure 3F,3G*). Thus, we selected STK31 for further study. Then, the relationship between STK31 expression and the clinical characteristics of PC patients were listed in *Table 3*. The expression of STK31 was significantly correlated with histological grade (P=0.0173).

The Kaplan-Meier survival curve also showed an inverse correlation between the expression level of STK31 and the prognosis of PC patients. Specifically, PC patients with high STK31 expression had a shorter OS (*Figure 3H*). Furthermore, results of qRT-PCR from PC tissues showed

Table 3 The association between STK31 expression and clinicopathological features of pancreatic cancer patients

Characteristics	Case	STK31 expression		P value
		Low (n=11)	High (n=19)	
Age at surgery (years)				0.4473
<60	11	5	6	
≥60	19	6	13	
Gender				0.0877
Male	17	4	13	
Female	13	7	6	
Tumor size (cm)				0.7048
≥3	15	6	9	
<3	15	5	10	
T grade				0.0877
T1+T2	13	7	6	
T3+T4	17	4	13	
Lymph node invasion				0.1804
Negative (N0)	20	9	11	
Positive (N1-N3)	10	2	8	
TNM stage				0.9791
I-II	19	7	12	
III-IV	11	4	7	
Histological grade				0.0173*
Low	16	9	7	
Middle-high	14	2	12	

*, P<0.05. TNM, tumor node metastasis.

a highly negative correlation between microRNA-543 and STK31 expression (*Figure 3I*). Therefore, the results suggest that STK31 may be the target gene of microRNA-543 and a prognostic marker of PC.

LINC00847 regulates the tumor development of PC and EMT in vitro by absorbing microRNA-543

To further investigate the novel function of microRNA-543, LncBase and starBase3.0 were utilized as bioinformatics tools to find possible lncRNA targets of microRNA-543. As shown in *Figure 4A*, 11 lncRNAs were obtained. Then, through comprehensive analysis and literature research, LINC00847 was selected as the target molecule, which was highly expressed in PC (*Figure 4B*). qRT-PCR analysis revealed that LINC00847 was obviously expressed in PC tissues and cells compared with normal tissues and H6C7 cells (*Figure 4C*,*4D*). Furthermore, when we collected the clinical data of the PC patients, it was found that the expression level of LINC00847 significantly correlated with the TNM stage of PC tissues (P=0.0344, *Table 4*).

Furthermore, results of qRT-PCR in PC tissues showed a highly negative correlation between microRNA-543 and LINC00847 expression (*Figure 4E*). Then, we transfected si-LINC00847 and oe-LINC00847 in BxPC-3 and PANC-1 cell lines and found that the transcription of microRNA-543 was decreased when LINC00847 was overexpressed, while the transcription of microRNA-543 was increased when LINC00847 was knocked down (*Figure 4F*). An assay for luciferase reporter activity was performed to confirm this interaction. BxPC-3 and PANC-1 cells expressing the WT LINC00847 reporter showed decreased luciferase activity in the microRNA-543 overexpression group, whereas cells expressing the MUT LINC00847 reporter showed no change in luciferase activity in the microRNA-543 perturbation group (*Figure 4G*). RIP and RNA-pull down assays were further applied to confirm the direct interaction between LINC00847 and microRNA-543, which demonstrated that LINC00847 directly binds to microRNA-543 (*Figure 4H*,*4I*). FISH assays showed that LINC00847 RNA was concentrated in the cytoplasm of PC cells (*Figure 4J*). Therefore, we can confirm that LINC00847 is the target gene of microRNA-543.

To further evaluate the function of LINC00847 in PC, BxPC-3 and PANC-1 cells were either transfected with si-LINC00847 to inhibit LINC00847 transcription or transfected with overexpression-LINC00847 to promote LINC00847 transcription. Compared with the control group, cell transfection was successful (*Figure 5A*). Then, CCK-8 and colony formation assays were utilized to measure the impact of LINC00847 overexpression on the growth of PC cells. We found that overexpression of LINC00847 enhanced PC cell growth in terms of cell number, while silencing LINC00847 inhibited PC cell growth (*Figure 5B*,*5C*). Moreover, to assess the invasion and migration of PC cells, the Transwell assay was performed. The results revealed that overexpression of LINC00847 substantially promoted PC cell invasion and migration, while knockdown of LINC00847 had opposite results (*Figure 5D*,*5E*). In addition, LINC00847 overexpression

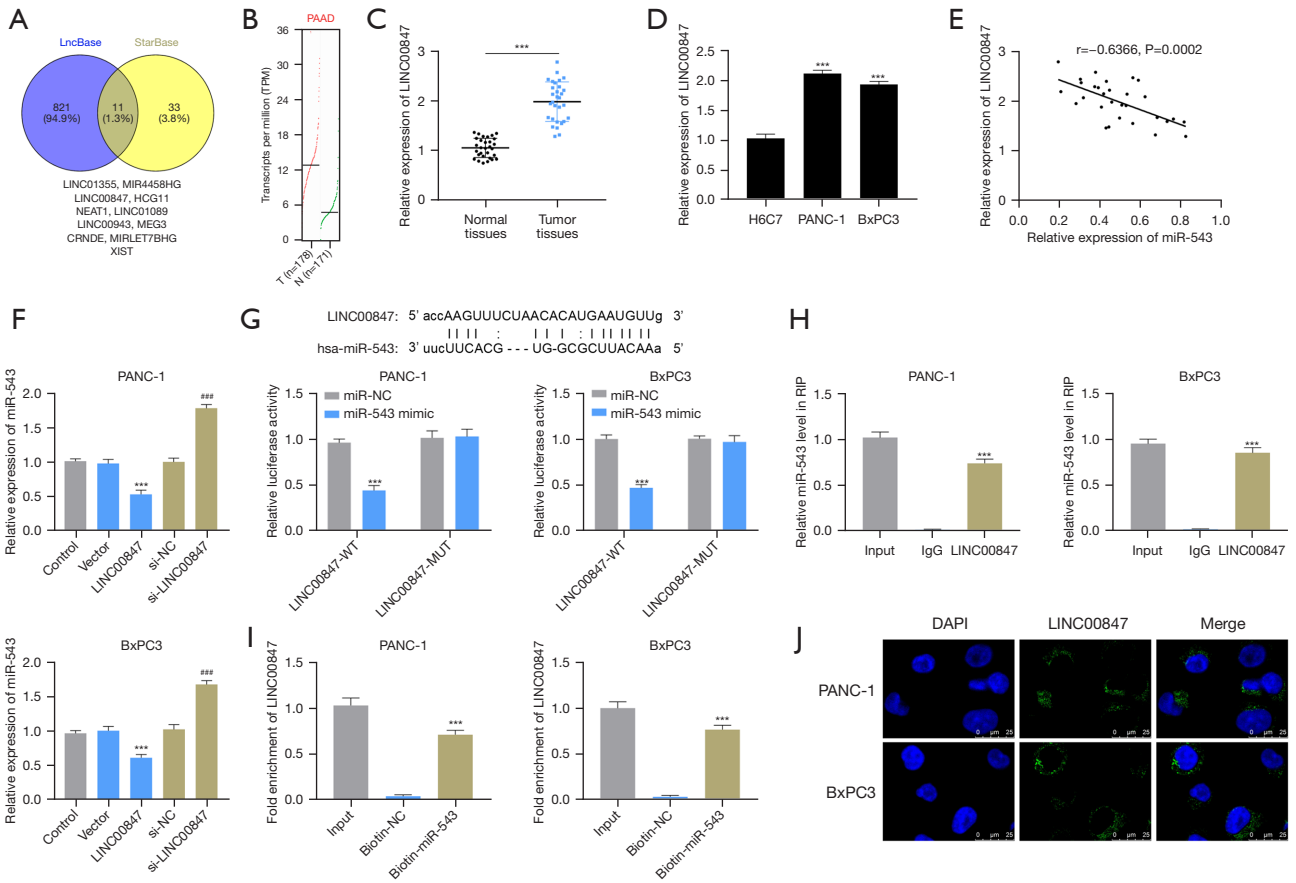

Figure 4 LINC00847 interacts with microRNA-543. (A) LINC00847 was identified as a target of microRNA-543 by Venn diagram analysis. (B) The transcription of LINC00847 by GEPIA2. Red means tumors, green means normal. (C,D) The mRNA transcription of LINC00847 was detected by qRT-PCR in PC tissue samples and cells. (E) Pearson correlation analysis of the correlation between LINC00847 and microRNA-543 expression based on PC tissues. (F) The transcription of microRNA-543 was detected by qRT-PCR after transfecting oe-LINC00847 or si-LINC00847 in BxPC-3 and PANC-1 cells. (G) The direct interaction between LINC00847 and microRNA-543 was investigated by the luciferase assay. (H) Using IgG antibodies, the RIP assay demonstrated a fold enrichment of LINC00847 and microRNA-543 in BxPC-3 and PANC-1 cells. (I) The levels of LINC00847 and microRNA-543 enrichment in BxPC-3 and PANC-1 cells were evaluated by the RNA pull down assay. (J) FISH was utilized to determine the subcellular localization of LINC00847 in BxPC-3 and PANC-1 cells. Original magnification 400×. ***, P<0.001, ###, P<0.001. PAAD, pancreatic adenocarcinoma; qRT-PCR, quantitative real-time polymerase chain reaction; PC, pancreatic cancer; RIP, RNA immunoprecipitation; FISH, fluorescence in-situ hybridization.

suppressed cell apoptosis, while LINC00847 knockdown accelerated cell apoptosis (*Figure 5F*). Finally, WB was applied to determine how LINC00847 affected the EMT process. According to *Figure 5G*, higher LINC00847 levels boosted N-cadherin, Vimentin, Slug, Snail, and TWIST protein expression in BxPC-3 and PANC-1 cells while inhibiting E-cadherin protein transcription, indicating the change from an epithelial to mesenchymal phenotype. The capacity of PC cells to develop the EMT feature was decreased through LINC00847 knockdown.

MicroRNA-543 controls the development of PC by the LINC00847/microRNA-543/STK31 axis

Furthermore, we explored whether microRNA-543 plays a carcinogenic role in PC through the LINC00847/microRNA-543/STK31 axis. The rescue experiments were constructed using microRNA-543 inhibitors, oe-STK31, or si-LINC00847. The data obtained from the qRT-PCR and WB assays showed that silencing LINC00847 reduced the transcriptional and translational level of STK31 in

Table 4 The association between LINC00847 expression and clinicopathological features of pancreatic cancer patients

Characteristics	Case	LINC00847 expression		P value
		Low (n=13)	High (n=17)	
Age at surgery (years)				0.5578
<60	11	4	7	
≥60	19	9	10	
Gender				0.2246
Male	17	9	8	
Female	13	4	9	
Tumor size (cm)				0.7125
≥3	15	6	9	
<3	15	7	8	
T grade				0.7851
T1+T2	13	6	7	
T3+T4	17	7	10	
Lymph node invasion				0.1927
Negative (N0)	20	7	13	
Positive (N1-N3)	10	6	4	
TNM stage				0.0344*
I-II	19	11	8	
III-IV	11	2	9	
Histological grade				0.1269
Low	16	9	7	
Middle-high	14	4	10	

*, P<0.05. TNM, tumor node metastasis.

BxPC-3 and PANC-1 cells, while the effects of silencing LINC00847 were reversed by microRNA-543 inhibitor or oe-STK31 (*Figure 6A,6B*).

Subsequently, we investigated whether microRNA-543 inhibitor or oe-STK31 might reverse the role of LINC00847 in PC cells. Inhibition of LINC00847 transcription resulted in the decreased cell number, invasion, and migration of PC cells as well as an increase in apoptosis, as determined by the CCK-8, colony formation, Transwell, and FCM assays. The inhibitor of microRNA-543 or oe-STK31 reversed the results (*Figure 6C-6G*).

WB analysis revealed that the transcription of mesenchymal markers N-cadherin, Snail, and TWIST were all reduced when LINC00847 was silenced; however, when co-transfection with microRNA-543 inhibitor or oe-STK31 was performed, the transcription of these markers was restored. In contrast to the transcription patterns of N-cadherin, Vimentin, Slug, Snail, and TWIST, the transcription pattern of E-cadherin was the inverse of that of the other proteins (*Figure 6H*). In this study, we hypothesized that microRNA-543 controlled the growth and development of PC cells through the LINC00847/microRNA-543/STK31 axis.

Discussion

PC is dubbed the "king of cancer" due to its aggressive biological nature and a dearth of viable treatments (24,25). As a result, it is critical to identify sensitive therapeutic targets or prognostic indicators to ensure that PC patients are receiving the most effective therapies possible.

We found that microRNA-543 was substantially downregulated and related to survival in PC by TCGA database, which suggested that microRNA-543 might be involved in the development of PC. Liu *et al.* reported that microRNA-543 inhibited cervical cancer growth and metastasis by targeting TRPM7 (5). Other studies also found that microRNA-543 can control the disease development of cervical cancer, colorectal cancer, non-small lung cancer, and prostate cancer (5,7,8,26). Wang *et al.* showed that LncRNA PVT1 controls TRPS1 transcription in breast cancer by absorbing microRNA-543 (27). Yuan *et al.* found that microRNA-543 was associated with the occurrence and prognosis of PC by targeting STK31 through a comprehensive bioinformatics analysis (22). However, they did not perform a thorough study, while we performed a deep study of the mechanism of this result. In our study, we confirmed that microRNA-543 promoted PC cell growth, invasion, migration, and EMT processes by targeting STK31.

Then, using a comprehensive bioinformatics study, we determined that LINC00847 may be the microRNA-543 target molecule. Additionally, we discovered that LINC00847 acted as an oncogene, promoting the cell growth, metastasis, invasion, and EMT of PC cells *in vitro*. Therefore, we built a competitive endogenous RNA (ceRNA) network of LINC00847/microRNA-543/STK31.

For the purpose of determining whether microRNA-543 promotes tumor progression through the LINC00847/microRNA-543/STK31 axis, rescue tests were designed and

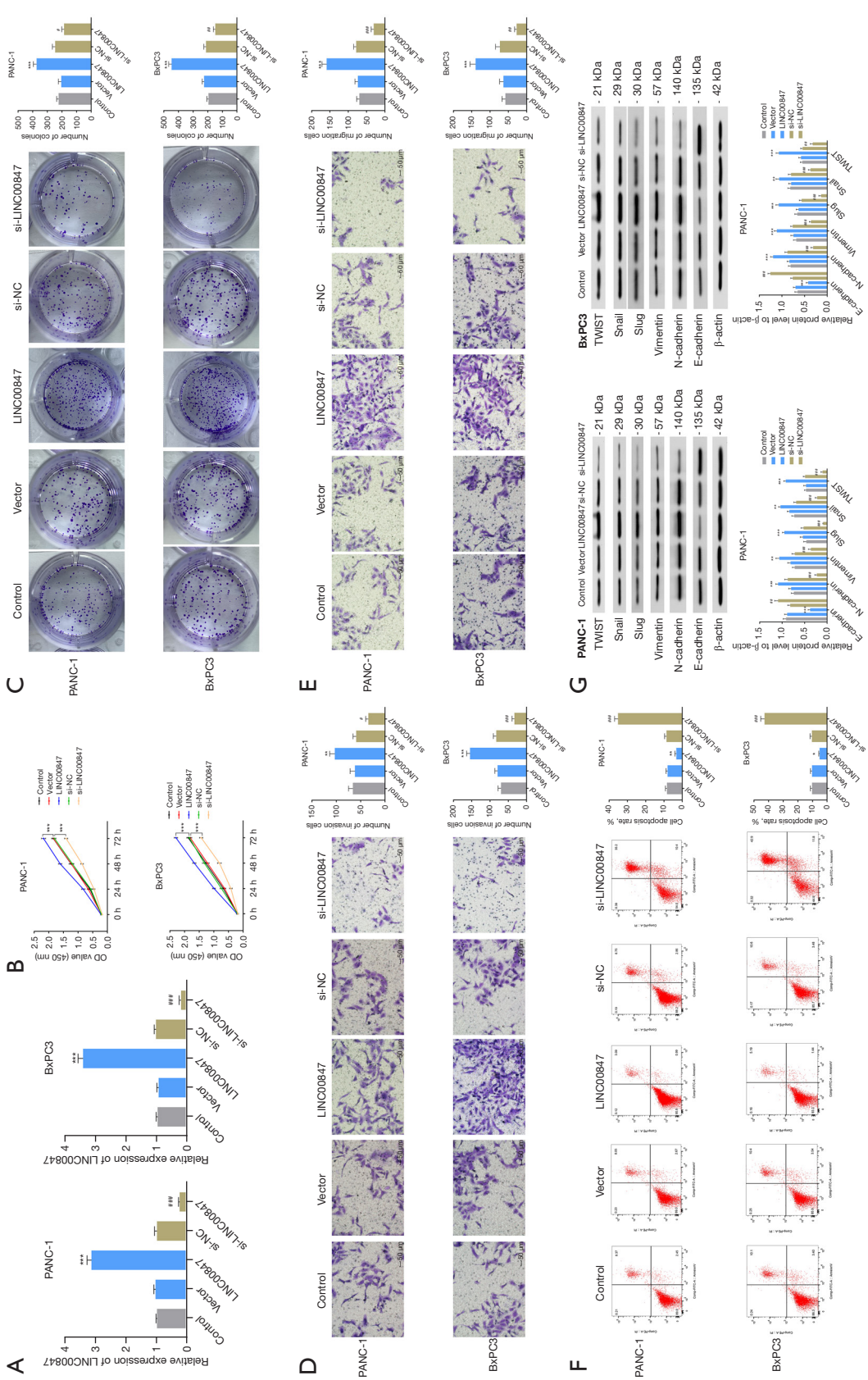

Figure 5 LINC00847 overexpression promoted PC cell growth and EMT in vitro. (A) The impact of stable transfection of PC cell lines was detected by the qRT-PCR assay. (B,C) The cell viability and growth of PC cells were analyzed by the CCK-8 and colony formation assays (dyed with crystal violet and taken pictures with Digital Camera). (D,E) Transwell assays were utilized to evaluate the migratory and invasive capacity of PC cell lines (dyed with crystal violet, 50 μm). (F) The apoptosis of PC cells was analyzed by the FCM assay. (G) The protein expression of N-cadherin, E-cadherin, Vimentin, Slug, Snail, and TWIST was detected in the LINC00847 overexpression and silencing groups by WB. *, P<0.05, **, P<0.01, ***, P<0.001, #, P<0.05, ##, P<0.01, ###, P<0.001. EMT, epithelial–mesenchymal transition; PC, pancreatic cancer; qRT-PCR, quantitative real-time polymerase chain reaction; FCM, flow cytometry; TWIST, twist family bHLH transcription factor 1; WB, Western blotting.

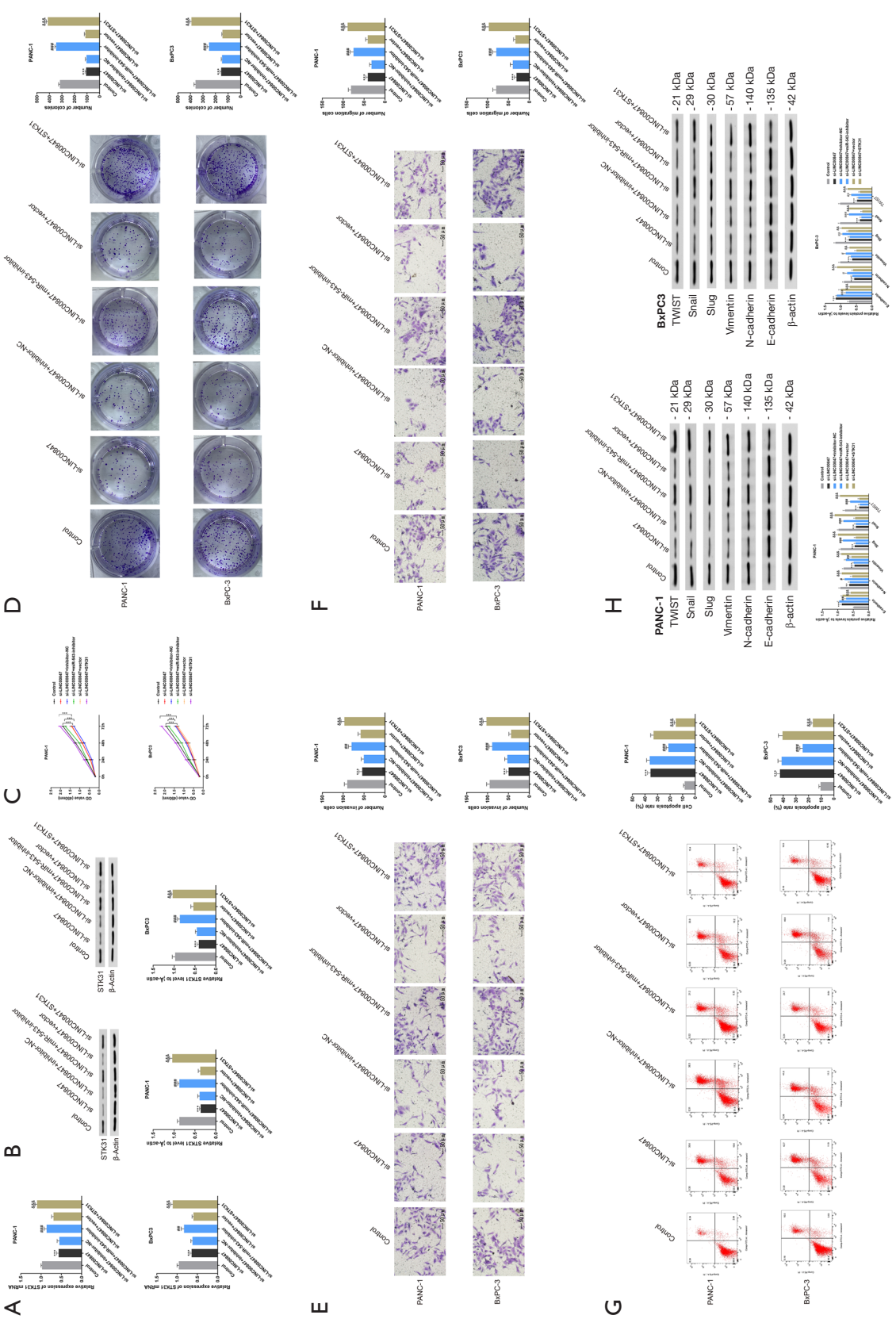

Figure 6 MicroRNA-543 controls the development and progression of PC cells by the LINC00847/microRNA-543/STK31 axis. (A,B) Results of qRT-PCR and WB analysis of STK31 levels in BxPC-3 and PANC-1 cells transfected with the NC or STK31 mimics, si-LINC00847, or oe-STK31. (C-G) The colony formation assay, CCK-8 assay, Transwell assay, and FCM were performed to assess the growth in cell number, migration, invasion, and apoptosis of PC cells that had been transfected with the indicated NC, mimics, si-LINC00847, or oe-STK31 (D) >50 cells, crystal violet-dyed; (E,F) crystal violet-dyed, 50 μm. (H) WB was utilized to detect the epithelial markers E-cadherin, N-cadherin, Vimentin, Slug, Snail, and TWIST in PC cells. The gathered data were reported as mean SD. **, P<0.01, ***, P<0.001, #, P<0.05, ##, P<0.01, ###, P<0.001, &&, P<0.01, &&&, P<0.001. qRT-PCR, quantitative real-time polymerase chain reaction; NC, normal control; FCM, flow cytometry; WB, western blotting; SD, standard deviation.

carried out using either the microRNA-543 inhibitor, si-LINC00847, or oe-STK31. When qRT-PCR and WB were used to analyze the results, it was discovered that decreasing LINC00847 transcription resulted in higher transcriptional and translational levels of STK31 in BxPC-3 and PANC-1 cells. At the same time, microRNA-543 inhibitors or oe-STK31 were found to be effective in reversing the consequences of decreased LINC00847 transcription.

Furthermore, we explored whether microRNA-543 inhibitors or oe-STK31 could reverse the effects of si-LINC00847 in BxPC-3 and PANC-1 cells, which was found to be effective. LINC00847 was found to inhibit the growth of BxPC-3 and PANC-1 cells, as well as their ability to invade and migrate in the Transwell and FCM assays. This was reversed by the microRNA-543 inhibitor or oe-STK31, which was found to inhibit the cell growth, invasion, and migration of BxPC-3 and PANC-1 cells.

Rescue experiments later indicated that LINC00847 had a negative impact on the oncogenic function of STK31. Also, we observed that decreasing LINC00847 transcription in BxPC-3 and PANC-1 cells resulted in a reduction of STK31 transcription in these cells. The function of STK31, on the other hand, was improved when the microRNA-543 inhibitor or oe-STK31 was added. As a result, our findings showed that LINC00847 acts as a ceRNA by harboring microRNA-543 and, as a result, neutralized the carcinogenic impact of microRNA-543 over the target gene STK31 during the growth of PC. Consequently, microRNA-543 has the potential to be a novel prospective biomarker for PC.

Unfortunately, there are some limitations to this research. Firstly, the validation was only performed *in vitro*, necessitating further *in vivo* studies with more clinical samples. Secondly, we only tested the mechanisms in cell lines, though they are incapable of reproducing *in vitro* or *in vivo* results in a reliable manner. Primary PC cells extracted from patient tissues or patient-derived xenograft models should be employed for additional validation.

Conclusions

In summary, we identified microRNA-543 as a possible treatment biomarker for PC. Our findings also revealed that LINC00847, and microRNA-543, had the ability to act as a ceRNA, mitigating the antitumor impact of microRNA-543 on the target gene STK31 during the course of PC progression. Our findings therefore suggest that microRNA-543 may be a novel biomarker of the development of PC in humans.

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Footnote

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Data Sharing Statement: Available at https://jgo.amegroups.com/article/view/10.21037/jgo-22-1017/dss

Conflicts of Interest: All authors have completed the ICMJE uniform disclosure form (available at https://jgo.amegroups.com/article/view/10.21037/jgo-22-1017/coif). The authors have no conflicts of interest to declare.

Ethical Statement: The authors are accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved. The study was conducted according to the Helsinki Declaration (as revised in 2013) and approval was obtained from the Ethics Committee of China-Japan Union Hospital of Jilin University (No. 20221014001). Before the beginning of the study, each patient signed written informed consent.

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