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LINC00941 promotes proliferation and metastasis of pancreatic adenocarcinoma by competitively binding miR-873-3p and thus upregulates ATXN2

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Abstract. – OBJECTIVE: Globally, the incidence and mortality of pancreatic adenocarcinoma (PAAD) have constantly increased. Long non-coding RNAs (lncRNAs) are considered as vital regulators in human cancers. This study aims to elucidate the role of LINC00941 in regulating PAAD progression and the molecular mechanism.

PATIENTS AND METHODS: Through database analyses, the expression pattern of LINC00941 in PAAD tissues and its prognostic value were uncovered. Its level in PAAD cell lines was detected by quantitative real-time polymerase chain reaction (qRT-PCR). After knockdown of LINC00941, proliferative and metastatic rates in BxPC-3 and PANC-1 cells were examined by cell counting kit-8 (CCK-8), 5-Ethynyl-2'-deoxyuridine (EdU) and transwell assay, respectively. The axis of LINC00941/miR-873-3p/ATXN2 was tested by Dual-Luciferase reporter assay and Pearson correlation test.

RESULTS: LINC00941 was abnormally upregulated in PAAD tissues, and linked to the prognosis. Knockdown of LINC00941 inhibited proliferative, migratory and invasive abilities in BxPC-3 and PANC-1 cells. MiR-873-3p was the target gene binding LINC00941, which was downregulated in PAAD tissues. Overexpression of miR-873-3p inhibited proliferative, migratory and invasive abilities in BxPC-3 and PANC-1 cells, and the inhibited trends were abolished by co-overexpression of LINC00941. Furthermore, ATXN2 was confirmed to be the target gene binding miR-873-3p, which was upregulated in PAAD tissues. It was negatively correlated to miR-873-3p and positively correlated to LINC00941.

CONCLUSIONS: LINC00941 is upregulated in PAAD tissues. It stimulates PAAD to proliferate and metastasize by competitively binding miR-873-3p and thus upregulates ATXN2.

Key Words:
Pancreatic adenocarcinoma, LINC00941, MiR-873-3p, ATXN2.

Introduction

Pancreatic cancer is a lethal malignant tumor of the digestive system. In the past 20 years, the mortality of pancreatic cancer ranks fourth in global cancers[1,2]. Pancreatic adenocarcinoma (PAAD) is the major subtype of pancreatic cancer, covering nearly 85% cases[3]. Surgery is the main strategy for the treatment of PAAD. Although great efforts have been made on improving the therapeutic efficacy of PAAD, its 5-year survival is extremely as low as 5%[4]. Therefore, it is urgent to illustrate the molecular mechanisms of the carcinogenesis and deterioration of PAAD, thus providing diagnostic and therapeutic markers.

Long non-coding RNAs (lncRNAs) are non-coding RNAs with longer than 200 nucleotides in transcripts[5]. Although lncRNAs lack the ability to encode proteins, they are extensively involved in the regulation of tumor process, transcriptional activities, epigenetic modifications, etc.[6]. Very recently, the vital functions of lncRNAs in tumor process have been gradually identified. It is reported that lncRNA NEF promotes migratory and invasive capacities in cervical squamous cell carcinoma by activating the TGF-β signaling[7]. Overexpression of lncRNA HNF1A-AS triggers nasopharyngeal carcinoma cells to proliferate and migrate[8]. LINC00675 is a prognostic factor for glioma, which is able to mediate growth and metastasis of glioma cells[9].

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MicroRNAs (miRNAs) are also a type of non-coding RNAs with 18-25 nucleotides long. They are responsible for negatively regulating target genes in the manner of complementary base pairing, thus inhibiting RNA translation or degrading them[10-12]. MiRNAs have a close relation to tumor cell functions. By binding FOXO1, miR-9 accelerates the metastasis of cervical cancer[13]. The proliferative rate of lung carcinoma cells can be suppressed by miR-129b[14]. MiR-1307 stimulates prostate cancer to proliferate *via* targeting FOXO3A[15].

A previous study has shown that LINC00941 is abnormally upregulated in gastric cancer cases[16]. Through database analysis, LINC00941 was found to be highly expressed in PAAD tissues. This study aims to uncover the biological function of LINC00941 in affecting the process of PAAD and the underlying mechanism.

Patients and Methods

GEPIA Dataset

Differential level of LINC00941 in PAAD tissues and its prognostic value on disease-free survival and overall survival of PAAD were analyzed using GEPIA dataset (http://GEPIA.cancer-pku.cn/index.html). The downloaded profile had 179 cases of PAAD and 171 cases of normal ones.

Clinical Samples

A total of 50 pairs of PAAD and adjacent normal tissues were collected from Taizhou People's Hospital. They were immediately frozen in liquid nitrogen *ex vivo*. All samples were stored at -80°C for RNA extraction. The pathological type and stage of tumor were determined following the UICC staging criteria. All participants were not treated with radiotherapy and chemotherapy before operation. Another kind of tumor that migrated to the pancreas was excluded. This study was approved by the Research Ethics Committee of Taizhou People's Hospital and complied with the Helsinki Declaration. Informed consent was obtained from patients prior to sample collection.

Cell Culture and Transfection

The pancreatic ductal epithelial cell line (HPDE6-CT) and PAAD cell lines (PANC-1, PK-9, BXPC-3, SW1990) were provided by American Type Culture Collection (ATCC; Manassas, VA, USA). Cells were cultivated in Roswell Park Memorial Institute-1640 (RPMI-1640; HyClone, South Logan, UT, USA) with 10% fetal bovine serum (FBS; HyClone, South Logan, UT, USA) in a humidified environment with 5% CO_2 at 37°C.

One day prior to transfection, cells were seeded in a 6-well plate and cultivated to higher than 60% of density. Cell transfection was routinely conducted using Lipofectamine 3000 (Invitrogen, Carlsbad, CA, USA).

Quantitative Real Time-Polymerase Chain Reaction (qRT-PCR)

RNA was reversely transcribed to complementary deoxyribose nucleic acid (cDNA) and subjected to qRT-PCR using the SYBR Premix Ex Taq II (TaKaRa, Dalian, China). Relative level was calculated using $2^{-\Delta\Delta CT}$ method. Primer sequences were as follows: LINC00941 F: 5′-ACCACTACACTCAGCCAAATAC-3′; R: 5′-GGCTATCAACTGTCTCCTTTAGAC-3′; miR-873-3p F: 5′-TTTGTGTGCATTTGCAGGAACT-3′; R: 5′-GAAGATTTGTGGGTGTTCCCG-3′; glyceraldehyde 3-phosphate dehydrogenase (GAPDH) F: 5′-ATGGGGAAGGTGAAGGTCG-3′; R: 5′-GGGGTCATTGATGGCAACAATA-3′; U6 F: 5′-CTCGCTTCGGCAGCACA-3′; R: 5′-ACGCTTCACGAATTTGCGT-3′.

Cell Counting Kit-8 (CCK-8) Assay

Cells were inoculated in a 96-well plate with 1×10^5 cells per well. At the appointed time points, absorbance value at 450 nm of each sample was recorded using the CCK-8 kit (RIBOBIO, Guangzhou, China) for plotting the viability curves.

5-Ethynyl-2′-Deoxyuridine (EdU) Assay

Cells were pre-inoculated in a 24-well plate (2×10^4 cells/well). They were incubated in 4% methanol for 30 min, followed by 10-min permeabilization in 0.5% TritonX-100 (Beyotime, Shanghai, China), and 30-min reaction in 400 μL of 1×ApollorR. Afterwards, cells were dyed in 4',6-diamidino-2-phenylindole (DAPI) for another 30 min. EdU-stained cells were calculated for calculating the percentage of positive EdU-stained cells (Beyotime, Shanghai, China).

Transwell

Transwell chambers (Millipore, Billerica, MA, USA) were inserted in each well of a 24-well plate, where 5×10^4 cells were applied in the upper layer of the chamber, and 600 μL of medium containing 10% FBS was applied in the bottom.

After 48-h incubation, cells in the bottom were reacted with 15-min methanol, 20-min crystal violet and captured using a microscope. Migratory cells were counted in 5 randomly selected fields per sample. Invasion assay was conducted using transwell chamber precoated with 100 μg Matrigel.

Dual-Luciferase Reporter Assay

Binding sites between two genes were predicted using online tools. Cells were seeded in the 24-well plate one day prior to co-transfection. They were co-transfected with Luciferase vectors and miR-873-3p mimics or negative control for 48 h. Luciferase activity was measured using the dual-luciferase reporter assay system (Promega, Madison, WI, USA).

Statistical Analysis

Data processing was conducted using Statistical Product and Service Solutions (SPSS) 16.0 (SPSS Inc., Chicago, IL, USA). Differences between two groups were analyzed by using the Student's *t*-test. The Kaplan-Meier curves followed by the log rank test were enrolled for the survival analysis. Comparison between multiple groups was done using One-way ANOVA test followed by Post-Hoc Test (Least Significant Difference). $p<0.05$ indicated the significant difference.

Results

Upregulation of LINC00941 in PAAD

By analyzing GEPIA database, LINC00941 was identified to be upregulated in PAAD tissues than normal ones (Figure 1A). Consistently, LINC00941 was abnormally upregulated in clinical samples of PAAD and PAAD cell lines (Figure 1B). Moreover, survival analysis showed that high level of LINC00941 was unfavorable to the disease-free survival and overall survival in PAAD patients (Figure 1C-1E).

Silence of LINC00941 Suppressed Proliferative and Metastatic Potentials in PAAD

LINC00941 level was silenced by transfection of either si-LINC00941#1 or si-LINC00941#2 in PANC-1 and BxPC-3 cells, and the latter one was used because of the better transfection efficacy (Figure 2A). After knockdown of LINC00941 in

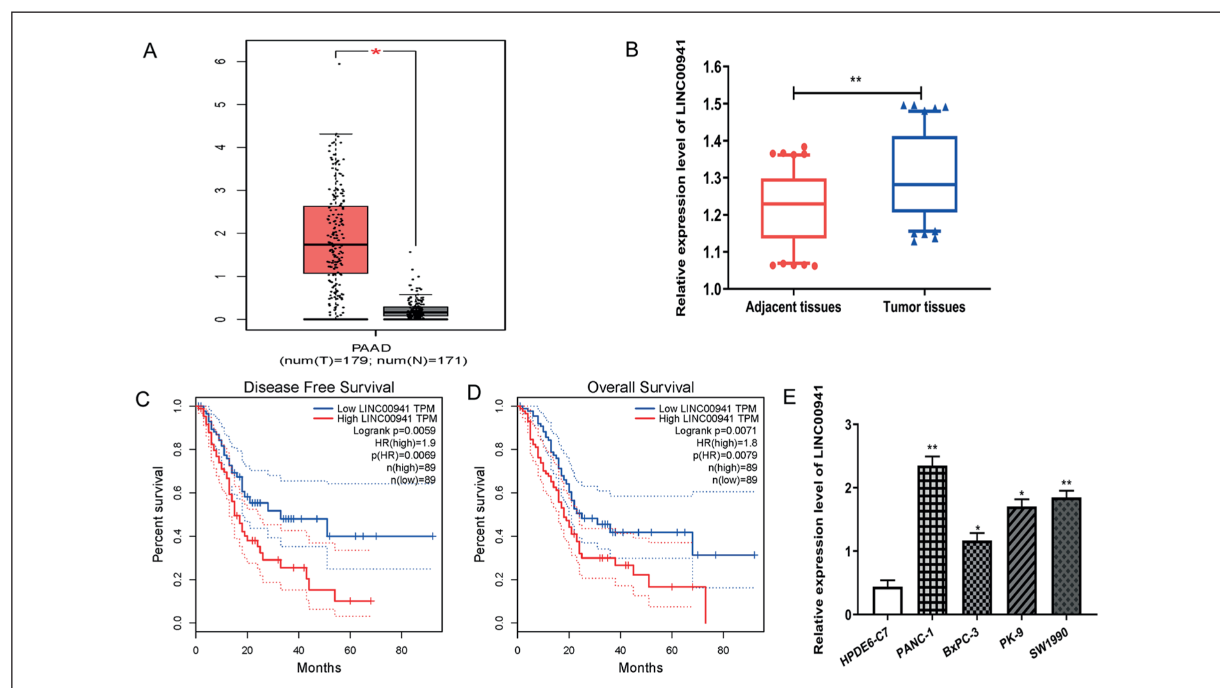

Figure 1. Upregulation of LINC00941 in PAAD. **A,** Differential level of LINC00941 in downloaded PAAD profile. **B,** Differential level of LINC00941 in PAAD tissues. **C,** Disease-free survival in PAAD patients with high or low level of LINC00941. **D,** Overall survival in PAAD patients with high or low level of LINC00941. **E,** LINC00941 level in PAAD cell lines. *$p<0.05$; **$p<0.01$.

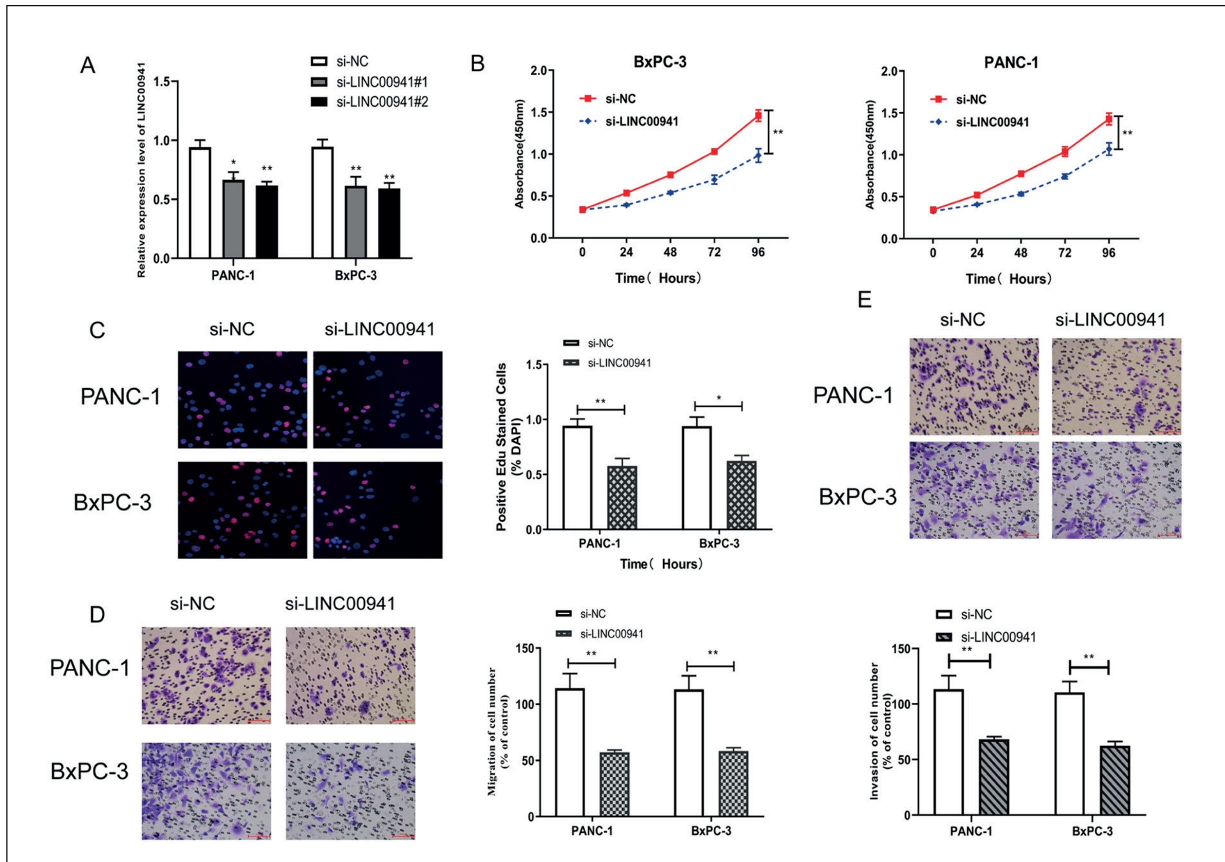

Figure 2. Silence of LINC00941 suppressed proliferative and metastatic potentials in PAAD. **A,** Transfection efficacy of si-LINC00941#1 and si-LINC00941#2 in PANC-1 and BxPC-3 cells. **B,** Viability in PANC-1 and BxPC-3 cells regulated by LINC00941. **C,** Percentage of EdU-stained cells in PANC-1 and BxPC-3 cells regulated by LINC00941 (magnification 200×). **D,** Migration in PANC-1 and BxPC-3 cells regulated by LINC00941 (magnification 200×). **E,** Invasion in PANC-1 and BxPC-3 cells regulated by LINC00941 (magnification 200×). *$p<0.05$; **$p<0.01$.

PAAD cells, viability and the percentage of EdU-stained cells were reduced, suggesting the suppressed proliferative ability (Figure 2B, 2C). In addition, transwell assay uncovered that knockdown of LINC00941 inhibited migratory and invasive abilities in PANC-1 and BxPC-3 cells (Figure 2D, 2E).

MiR-873-3p Was the Target Gene of LINC00941

A binding site in the 3'UTR of miR-873-3p and LINC00941 was predicted using bioinformatic software (Figure 3A). Subsequently, Dual-Luciferase reporter assay showed that miR-873-3p was able to bind the wild-type LINC00941 vector, rather than the mutant-type one, confirming the binding between miR-873-3p and LINC00941 (Figure 3B). MiR-873-3p was downregulated in PAAD tissues, and negatively correlated to LINC00941 level (Figure 3C, 3D). As expected, transfection of si-LINC00941 upregulated miR-873-3p in PANC-1 and BxPC-3 cells (Figure 3E).

MiR-873-3p Was Responsible for the Regulatory Effect of LINC00941 on PAAD Phenotypes

We thereafter explored the potential influences of miR-873-3p on PAAD phenotypes. Transfection of miR-873-3p mimics effectively upregulated miR-873-3p in PAAD cells. Notably, the upregulated miR-873-3p in PANC-1 and BxPC-3 cells overexpressing miR-873-3p was inhibited by co-overexpression of LINC00941 (Figure 4A). Later, viability and the percentage of EdU-stained cells were reduced by overexpression of miR-873-

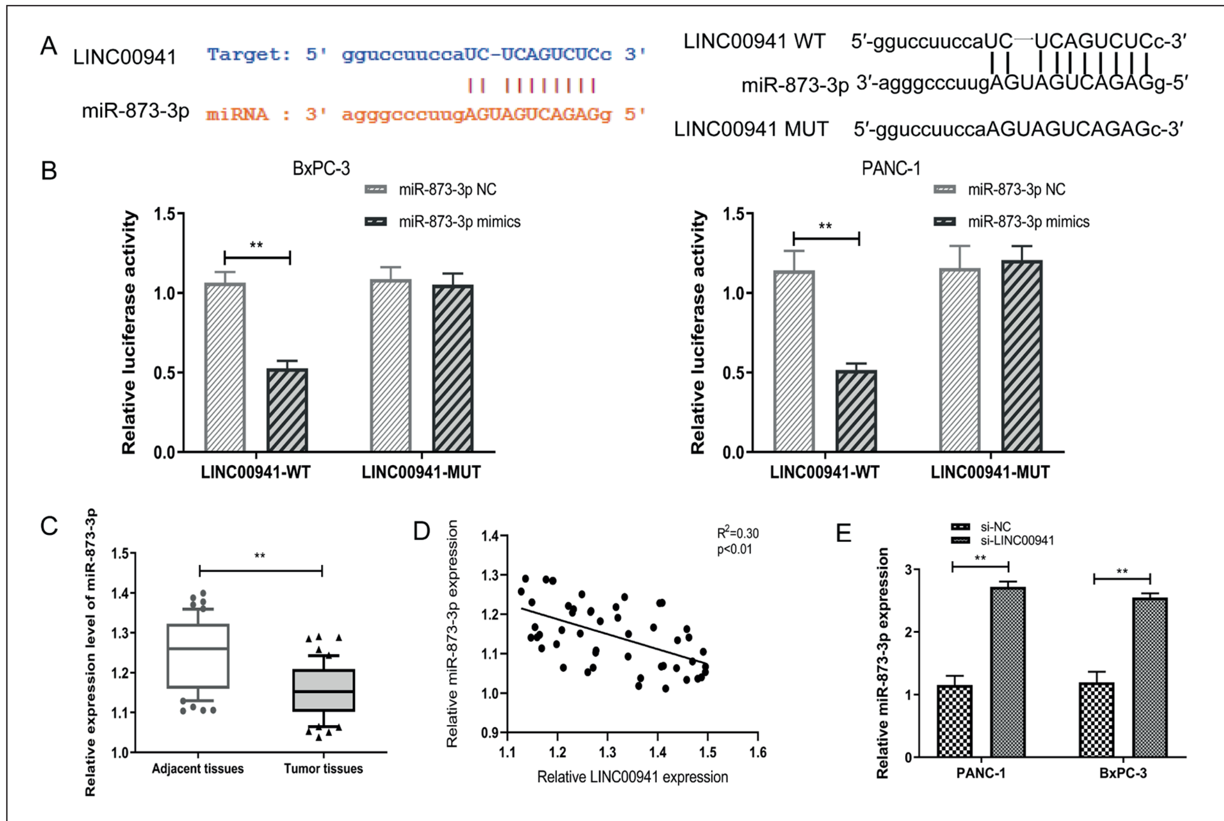

Figure 3. MiR-873-3p was the target gene of LINC00941. **A,** A binding site in the 3'UTR of LINC00941 and miR-873-3p. **B,** Luciferase activity in co-transfected BxPC-3 and PANC-1 cells. **C,** Differential level of miR-873-3p in PAAD tissues. **D,** A negative correlation between LINC00941 and miR-873-3p. **E,** MiR-873-3p level in PANC-1 and BxPC-3 cells regulated by LINC00941. $^{**}p<0.01$.

3p in PAAD cells, and interestingly, the declined proliferative potential was suppressed by overexpressed LINC00941 (Figure 4B, 4C). Similarly, suppressed migratory and invasive rates in PAAD cells overexpressing miR-873-3p were abolished by co-overexpression of LINC00941 (Figure 4D, 4E).

ATXN2 Was the Downstream Gene of MiR-873-3p

We searched downstream targets of miR-873-3p using bioinformatic tools, and ATXN2 was selected after cross-match (Figure 5A). The binding site in the 3'UTR of ATXN2 and miR-873-3p was predicted (Figure 5B). In the same way, ATXN2 was verified to be the downstream gene that was directly bound to miR-873-3p (Figure 5C). In addition, ATXN2 was upregulated in PAAD tissues (Figure 5D). It had a negative correlation to miR-873-3p level, and a positive correlation to LINC00941 level (Figure 5E).

Discussion

Pancreatic cancer is featured by extremely strong invasiveness and high mortality[17]. Most patients are diagnosed in the advanced stage of pancreatic cancer, with a very poor prognosis[18]. With the rapid increase in the incidence of pancreatic cancer, it is estimated that in 2030, it will become the second most-common malignancy in the world[19]. Previously, the biological function of LINC00941 has been discovered in gastric cancer[20]. By analyzing an online database, it is identified that LINC00941 was abnormally upregulated in PAAD tissues. Based on this finding, this study focused on the exploration of the biological function of LINC00941 in affecting PAAD process and the possible mechanism.

Through a series of *in vitro* experiments, it is demonstrated that LINC00941 exerted an oncogenic role in PAAD process by promoting proliferative and metastatic potentials of cancer cells. Moreover, abnormally upregulated LINC00941

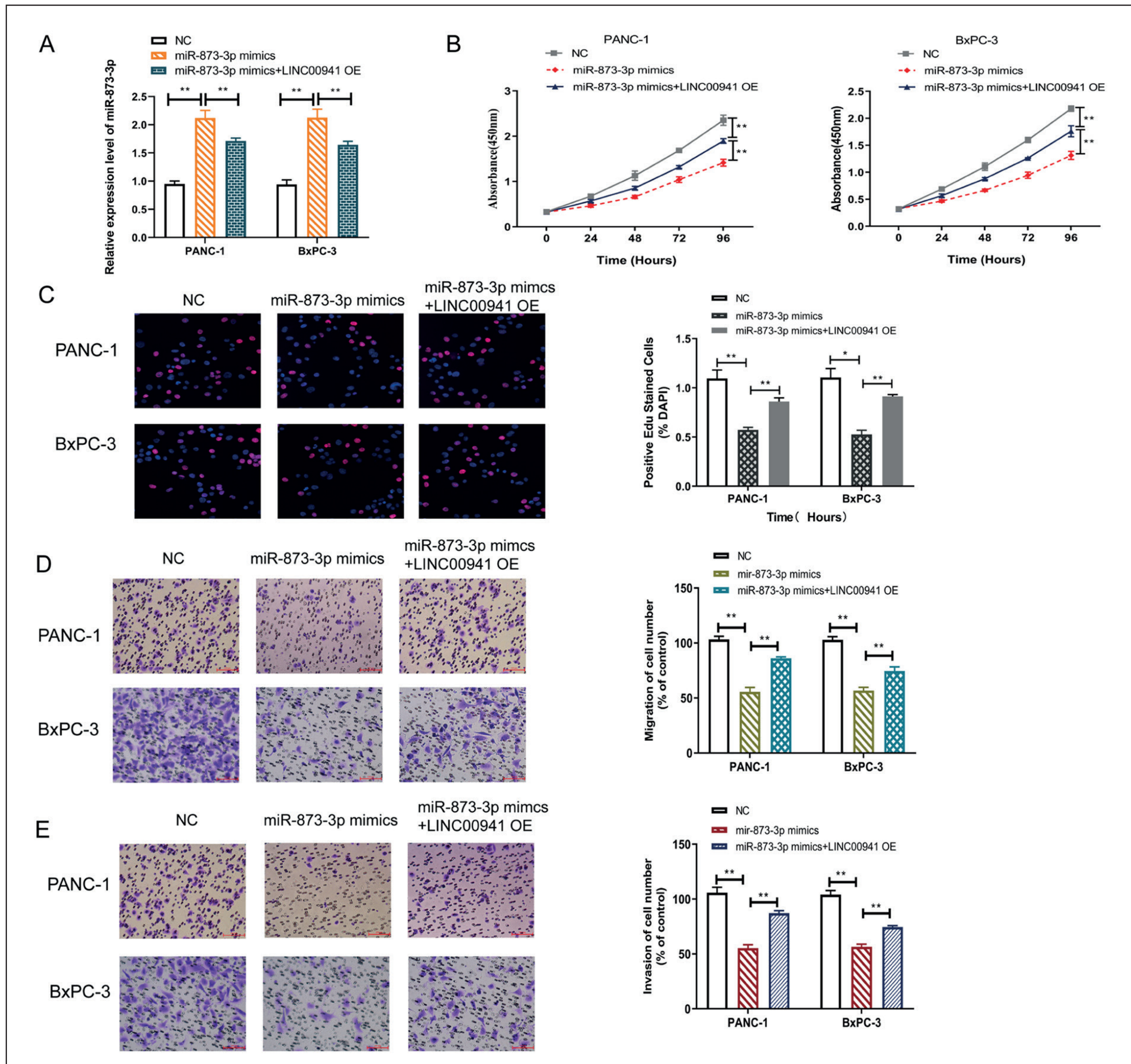

Figure 4. MiR-873-3p was responsible for the regulatory effect of LINC00941 on PAAD phenotypes. **A,** Transfection efficacy of miR-873-3p mimics in PANC-1 and BxPC-3 cells. **B,** Viability in PANC-1 and BxPC-3 cells co-regulated by LINC00941 and miR-873-3p. **C,** Percentage of EdU-stained cells in PANC-1 and BxPC-3 cells co-regulated by LINC00941 and miR-873-3p (magnification 200×). **D,** Migration in PANC-1 and BxPC-3 cells co-regulated by LINC00941 and miR-873-3p (magnification 200×). **E,** Invasion in PANC-1 and BxPC-3 cells co-regulated by LINC00941 and miR-873-3p (magnification 200×). *$p<0.05$; **$p<0.01$.

was closely linked to a poor prognosis of PAAD. In the previous study, lncRNAs were reported function as a ceRNA for miRNA to regulating the target genes. In our study, miR-873-3p was confirmed to be the target gene binding LINC00941, showing a negative correlation between each other. MiR-873-3p, notably, could partially abolish the influence of LINC00941 on malignant phenotypes of PAAD.

MiRNAs are post-transcriptional regulators for gene expressions[21]. They are functional in cell behaviors by targeting various genes[22-24]. In our research, ATXN2 was detected to be the direct target of miR-873-3p. ATXN2 is a 124-kDa stress protein expressed in specific groups of neurons and hepatocytes[25]. As located in endoplasmic reticulum and Golgi apparatus, ATXN2 is able to mediate mRNA maturation, translation and endocytosis[26]. Our findings uncovered that ATXN2 was upregulated in PAAD tissues, which was negatively correlated to miR-873-3p level, but positively correlated to LINC00941 level. It

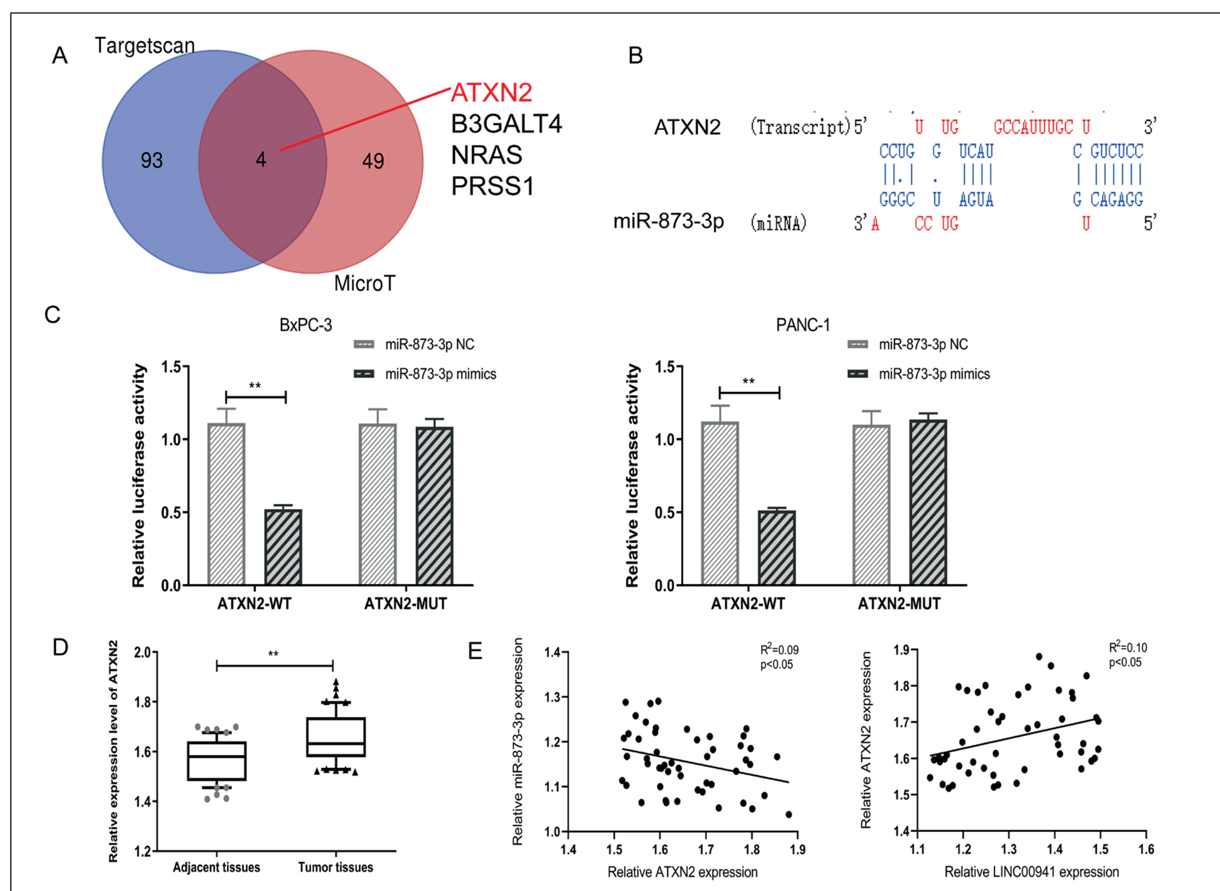

Figure 5. ATXN2 was the downstream gene of miR-873-3p. **A,** Prediction of potential targets of miR-873-3p and the cross-matched results. **B,** A binding site in the 3'UTR of miR-873-3p and ATXN2. **C,** Luciferase activity in co-transfected BxPC-3 and PANC-1 cells. **D,** Differential level of ATXN2 in PAAD tissues. **E,** A negative correlation between miR-873-3p and ATXN2, and a positive correlation between LINC00941 and ATXN2; *$p<0.05$; **$p<0.01$.

is identified that LINC00941 stimulated PAAD cells to proliferate and metastasize by activating the miR-873-3p/ATXN2 axis. In our study, it is the first time to report the role of LINC00941 in PAAD and provide a new target for the treatment in the future.

Conclusions

In sum, LINC00941 is upregulated in PAAD tissues. It stimulates PAAD to proliferate and metastasize by competitively binding miR-873-3p and thus upregulates ATXN2.

Conflict of Interest

The Authors declare that they have no conflict of interests.

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