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Circ_0005273 induces the aggravation of pancreatic cancer by targeting KLF12

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Abstract. – OBJECTIVE: The purpose of this study was to explore the potential influences of circ_0005273 and its downstream target KLF12 on the progression of pancreatic cancer.

PATIENTS AND METHODS: Relative levels of circ_0005273 and KLF12 in paired pancreatic cancer tissues and normal tissues were detected by quantitative real-time polymerase chain reaction (qRT-PCR). Then, the differences in clinical indicators and prognosis (overall survival and progression-free survival) between pancreatic cancer patients expressing high and low levels of circ_0005273 were compared. After knockdown of circ_0005273 in AsPC-1 and CFPAC-1 cells, viability and migratory ability were assessed by cell counting kit-8 (CCK-8), transwell and wound healing assays. The regulatory effect of circ_0005273 on KLF12 was determined through Western blotting assay. Finally, the interaction between circ_0005273 and KLF12 was tested by dual-luciferase reporter assay.

RESULTS: It was found that circ_0005273 was upregulated in pancreatic cancer tissues than that in normal tissues. Besides, pancreatic cancer patients expressing a high level of circ_0005273 had higher incidence rates of lymphatic metastasis and distant metastasis, as well as poor prognosis. Knockdown of circ_0005273 weakened the proliferative and migratory abilities of AsPC-1 and CFPAC-1 cells. KLF12 was the target gene binding to circ_0005273, showing a negative expression correlation between each other. Moreover, the protein level of KLF12 was downregulated by knockdown of circ_0005273. KLF12 was able to abolish the regulatory effects of circ_0005273 on the phenotypes of pancreatic cancer cells.

CONCLUSIONS: Circ_0005273 drives proliferative and migratory abilities of pancreatic cancer cells via activating the KLF12, and it is able to predict lymphatic metastasis, distant metastasis and prognosis in pancreatic cancer patients.

Key Words:
Circ_0005273, KLF12, Pancreatic cancer, Cancer aggravation.

Introduction

Pancreatic cancer is a highly malignant tumor in the digestive system and its prognosis is extremely poor[1,2]. The incidence of pancreatic cancer gradually increases because of high-fat diet, increased smoking population, environmental pollutions, etc.[3,4]. In China, its incidence rises at 3-4% per year, and it becomes the fifth killer in cancer death[4,5]. Clinical symptoms of pancreatic cancer in the early stage are atypical. Middle stage or advanced pancreatic cancer is usually accompanied by local infiltration or distant metastasis, leading to a low surgical resectability and unsatisfactory outcomes of chemotherapy and radiotherapy[6,7]. The postoperative 1-year survival of pancreatic cancer is lower than 15% and the 5-year survival is only about 3%[8,9]. Therefore, screening, diagnosis and treatment of pancreatic cancer as early as possible are well concerned[10,11].

CircRNAs are non-coding RNAs lacking 3' and 5' ends, which are formed by a covalently closed loop and produced by pre-mRNAs[12,13]. Functionally, circRNAs exert the sponge effect on miRNAs, modulate transcription or splicing, and interact with RNA-binding proteins[14,15]. Owing to the special structure, circRNAs are more stable than linear RNAs, which are resistant to exonucleases. As a result, circRNAs may be novel diagnostic hallmarks for human diseases[15-17]. By directly or indirectly mediating miRNA expressions, circRNAs are able to regulate their activities and functions[18,19]. Through database analysis and literature review, circ_0005273 is highly expressed in many types of tumors, and its level is relevant to tumor grade and prognosis[20,21]. In this study, thus, pancreatic cancer tissues were collected for detecting the differential level of circ_0005273, and its biological functions in regulating pancreatic cancer progression were subsequently explored.

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Patients and Methods

Pancreatic Cancer Samples

During surgical procedure, 56 pairs of pancreatic cancer and normal tissues were collected. None of these patients were treated by preoperative medication and/or radiotherapy. Resected samples were independently confirmed by two experienced pathologists and stored at -80°C. Tumor node metastasis (TNM) stage and histological classification of pancreatic cancer were defined according to the criteria proposed by UICC/AJCC. Inclusion criteria: patients with no severe diseases in other organs and those undergoing no preoperative chemotherapy and post-operative radiotherapy. Exclusion criteria: patients complicated with other malignancies, those with mental disease, those complicated with myocardial infarction, heart failure or other chronic diseases, or those previously exposed to radioactive rays. This investigation was approved by the Ethics Committee of the Affiliated Yantai Yuhuangding Hospital of Qingdao University and informed consent was obtained from each subject.

Cell Lines and Reagents

Pancreatic cancer cell lines (AsPC-1, PANC-1, MIA PaCa-2, CFPAC-1, BxPC-3) and the pancreatic ductal epithelial cell line (HPNE) were purchased from American Type Culture Collection (ATCC) (Manassas, VA, USA). They were cultured in Dulbecco's Modified Eagle's Medium (DMEM) (Gibco, Rockville, MD, USA) supplemented with 10% fetal bovine serum (FBS) (Gibco, Rockville, MD, USA), 100 U/mL penicillin and 100 μg/mL streptomycin in a humidified incubator with 5% CO_2 at 37°C. When cell confluence reached 80-90%, cell passage was conducted using 1×trypsin+EDTA (ethylenediaminetetraacetic acid).

Transfection

Transfection plasmids, including sh-circ_0005273, sh-NC, si-KLF12, si-NC, pcDNA-KLF12 and pcDNA-NC, were constructed by GenePharma (Shanghai, China). Cells were cultured to 30-60% density and transfected using Lipofectamine™2000 (Invitrogen, Carlsbad, CA, USA). After 48 h, transfected cells were collected for functional experiments.

Cell Proliferation Assay

Transfected cells were inoculated in the 96-well plate with 2×10^3 cells/well. Six replicates were set in each group. On day 1, 2, 3 and 4, 10 μL of cell counting kit-8 (CCK-8) solution (Dojindo Molecular Technologies, Kumamoto, Japan) was added per well and incubated for 4 h, respectively. At last, optical density at 490 nm was measured using a microplate reader.

Transwell Migration Assay

Transfected cells were prepared into suspension with 5×10^5 cells/mL. 200 μL of suspension was added on the top of transwell chambers (Millipore, Billerica, MA, USA), which were pre-inserted in a 24-well plate. On the bottom, 600 μL of medium containing 10% FBS was applied. After 48-h incubation, transwell chambers were taken out. Cells in the bottom were subjected to methanol fixation for 15 min, and crystal violet (0.2%) staining for 20 min. Finally, migratory cells were counted in 5 randomly selected fields per sample.

Wound Healing Assay

Cells were prepared into suspension with 5×10^5 cells/ml and implanted in 6-well plates. Until 90% of cell attachment, an artificial wound was made using a sterilized pipette tip. Cells were washed in phosphate-buffered saline (PBS) for 2-3 times and cultured in the medium containing 1% FBS. 24 hours later, wound closure was captured for calculating the percentage of wound healing.

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

RNAs were extracted from pancreatic cancer cells and tissues using TRIzol (Invitrogen, Carlsbad, CA, USA) and reversely transcribed using Primescript RT Reagent (TaKaRa, Otsu, Shiga, Japan). Then, qRT-PCR was conducted using SYBR®Premix Ex Taq™ (TaKaRa, Otsu, Shiga, Japan) on the StepOne Plus Real-time PCR system (Applied Biosystems, Foster City, CA, USA). Relative level was calculated by the $2^{-\Delta\Delta Ct}$ method. circ_0005273: forward: 5'-AATGCCTGTGAACCCATAGTG-3', reverse: 5'-CTGACAGCATGAGCATCCCT-3'; KLF12: forward: 5'-TTTCCTGAGAACTGCAGAGAGC-3', reverse: 5'-GTCACATTGATCCTGAACAGAAG-3'; glyceraldehyde 3-phosphate dehydrogenase (GAPDH): forward: 5'-GGAGCGAGATCCCTCCAAAAT-3', reverse: 5'-GGCTGTTGTCATACTTCTCATGG-3'.

Western Blotting

Cells were lysed on ice for 30 min and centrifuged at 4°C, 14000×g for 15 min for isolating

proteins and electrophoresed. Next, protein samples were loaded on polyvinylidene difluoride (PVDF) membranes (Millipore, Billerica, MA, USA). Subsequently, non-specific antigens were blocked in 5% skim milk for 2 hours. Primary and secondary antibodies were applied for indicated time. Band exposure and analyses were finally conducted.

Dual-Luciferase Reporter Assay

Binding sequences in 3' untranslated region (3'UTR) of circ_0005273 and KLF12 were predicted online and inserted into pmirGLO, which was named as pmirGLO-circ_0005273-WT. Meanwhile, the mutant sequences were similarly inserted to generate pmirGLO-circ_0005273-MUT. pmirGLO was co-transfected into AsPC-1 and CFPAC-1 cells with either pcDNA-NC or pcDNA-KLF12. Luciferase activity (Promega, Madison, WI, USA) was finally measured at 48 h.

Statistical Analysis

Statistical analysis was conducted by GraphPad Prism 5 V5.01 (La Jolla, CA, USA). Differences between groups were detected using the Student's *t*-test. Differences in clinical indicators and prognosis between pancreatic cancer patients expressing high and low levels of circ_0005273 were examined by Chi-square test. Each experiment was repeated in triplicate and data were expressed as mean ± standard deviation. Kaplan-Meier curves were depicted for survival analysis. $p<0.05$ was considered as statistically significant.

Results

Circ_0005273 Was Highly Expressed in Pancreatic Cancer

A total of 56 paired pancreatic cancer tissues were collected. It was shown that circ_0005273 was upregulated in pancreatic cancer tissues than normal tissues (Figure 1A). Meanwhile, it was highly expressed in pancreatic cancer cell lines as well (Figure 1B). Notably, AsPC-1 and CFPAC-1 cells had the highest abundance of circ_0005273, which were selected for generating a circ_0005273 knockdown model.

Circ_0005273 Predicted Metastasis and Prognosis in Pancreatic Cancer Patients

Based on the mRNA level of circ_0005273 in 56 pancreatic cancer tissues, recruited patients were divided into high-level circ_0005273 group and low-level circ_0005273 group, respectively. Chi-square analysis was performed to assess the differences in age, gender, tumor staging and metastasis incidence between the two groups. The results identified that circ_0005273 level was significantly correlated with the incidences of lymphatic metastasis and distant metastasis in pancreatic cancer (Table I). Meanwhile, a higher level of circ_0005273 was detected in pancreatic cancer patients with lymphatic metastasis or distant metastasis than those with no metastasis, further supporting our findings (Figure 1C). In addition, overall survival and progression-free survival were worse in high-level circ_0005273 group than in low-level circ_0005273 group as Kaplan-Meier curves revealed (Figure 1D). The AUC value of 0.817 (95% CI=0.694-0.894) by the ROC curve was obtained, suggesting the diagnostic value of circ_0005273 in patients with pancreatic cancer.

Knockdown of Circ_0005273 Inhibited Proliferative and Migratory Capacities of Pancreatic Cancer Cells

Transfection of sh-circ_0005273 effectively downregulated circ_0005273 in AsPC-1 and CFPAC-1 cells (Figure 2A). Compared with those transfected with sh-NC, viability was reduced in AsPC-1 and CFPAC-1 cells with circ_0005273 knockdown (Figure 2B). Migratory change in pancreatic cancer cells influenced by circ_0005273 was assessed by transwell assay and wound healing assay. Transfection of sh-circ_0005273 reduced migratory cell number and wound closure percentage (Figure 2C, D). To sum up, circ_0005273 was able to promote proliferative and migratory capacities of pancreatic cancer cells.

Circ_0005273 Bound to KLF12

Bioinformatics analysis suggested the interaction between circ_0005273 and KLF12. Western blotting analyses uncovered that the protein levels of KLF12 were markedly downregulated in AsPC-1 and CFPAC-1 cells transfected with sh-circ_0005273 (Figure 3A). Besides, KLF12 was detected to be lowly expressed in pancreatic cancer tissues (Figure 3B). As expected, its level was negatively correlated with circ_0005273 (Figure 3C). To test the binding between KLF12 and circ_0005273, binding sequences in 3'UTR of circ_0005273 and KLF12 were inserted into pmirGLO, which was named as pmirGLO-

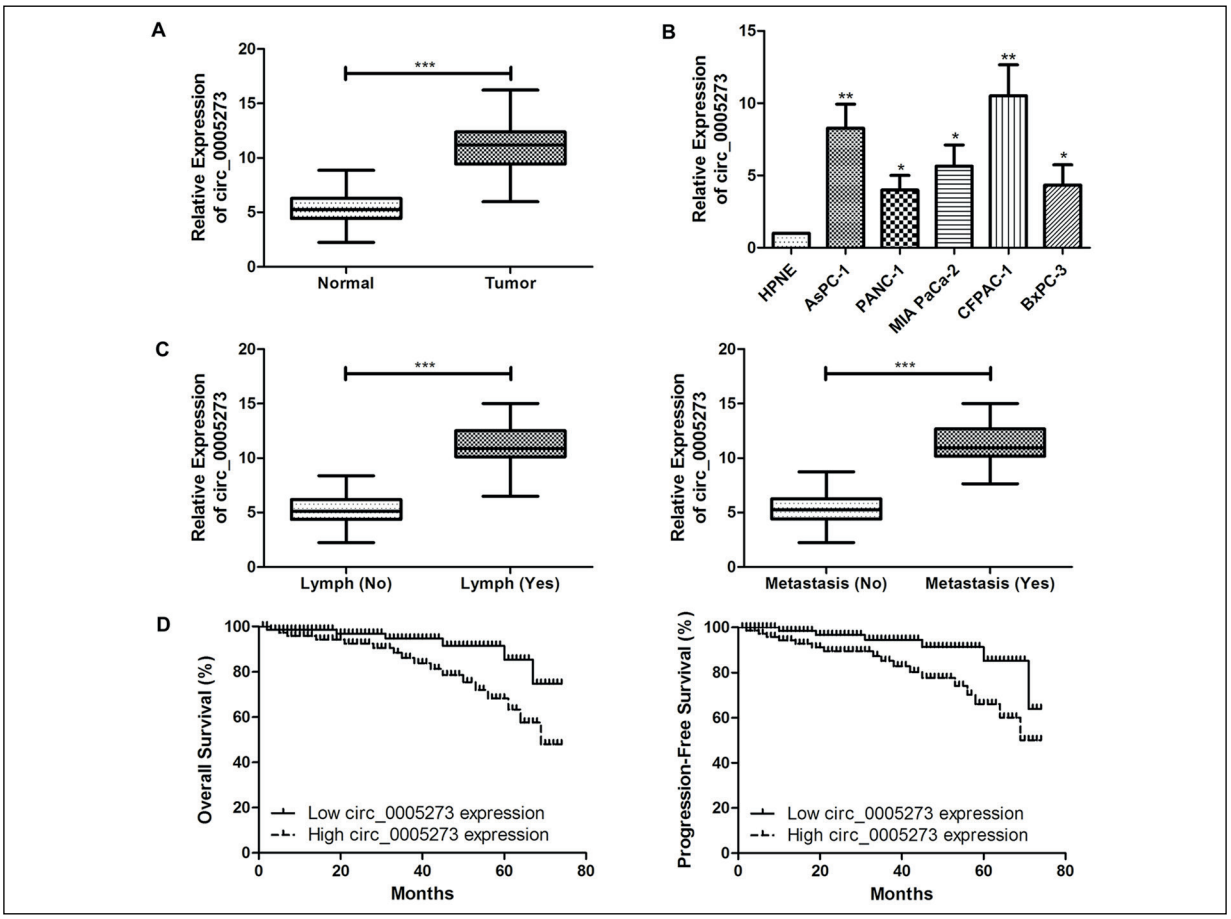

Figure 1. Circ_0005273 was highly expressed in pancreatic cancer. **A,** Differential expressions of circ_0005273 in pancreatic cancer tissues and normal tissues; **B,** Circ_0005273 levels in pancreatic cancer cell lines; **C,** Circ_0005273 levels in pancreatic cancer patients either with lymphatic metastasis, distant metastasis or not; **D,** Overall survival and progression-free survival in pancreatic cancer patients expressing high or low level of circ_0005273. Data were expressed as mean±SD. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$.

Table I. Association of circ_0005273 expression with clinicopathologic characteristics of pancreatic cancer.

Parameters	No. of cases	circ_0005273 expression		*p*-value
		Low (%)	High (%)	
Age (years)				0.984
< 60	22	13	9	
≥ 60	34	20	14	
Gender				0.656
Male	20	11	9	
Female	36	22	14	
Lymph node metastasis				0.049
No	33	23	10	
Yes	23	10	13	
Distance metastasis				0.023
No	32	23	9	
Yes	24	10	14	
Tumor size (cm)				0.135
< 4	31	21	10	
≥ 4	25	12	13	
T stage				0.592
T1-T2	34	21	13	
T3-T4	22	12	10	

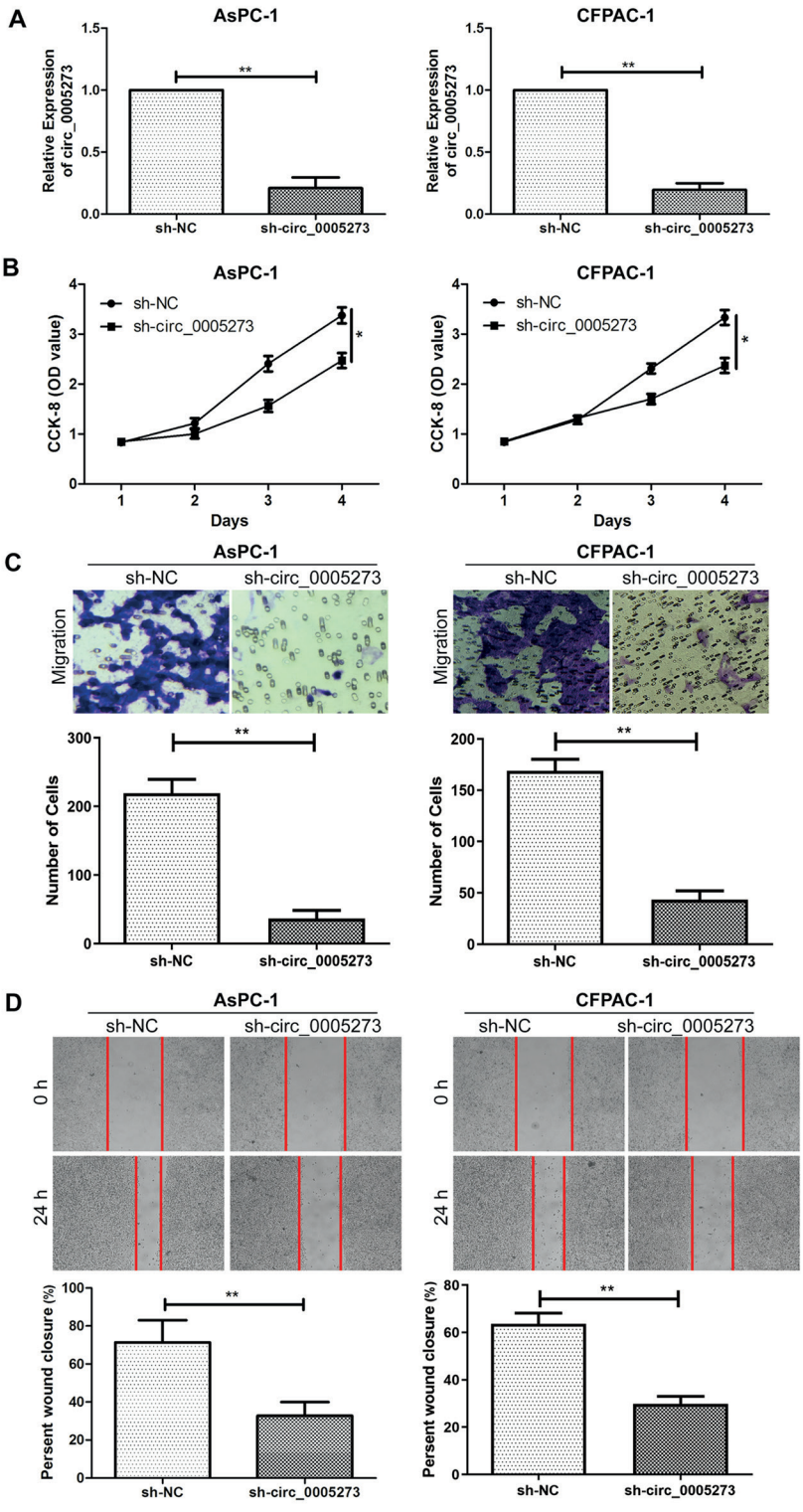

Figure 2. Knockdown of circ_0005273 inhibited proliferative and migratory capacities of pancreatic cancer cells. **A,** Transfection of sh-circ_0005273 in AsPC-1 and CFPAC-1 cells; **B,** Viability in AsPC-1 and CFPAC-1 cells transfected with sh-NC or sh-circ_0005273; **C,** Migration in AsPC-1 and CFPAC-1 cells transfected with sh-NC or sh-circ_0005273 (magnification: 40×); **D,** Wound closure AsPC-1 and CFPAC-1 cells transfected with sh-NC or sh-circ_0005273 (magnification: 40×). Data were expressed as mean±SD. *$p < 0.05$, **$p < 0.01$.

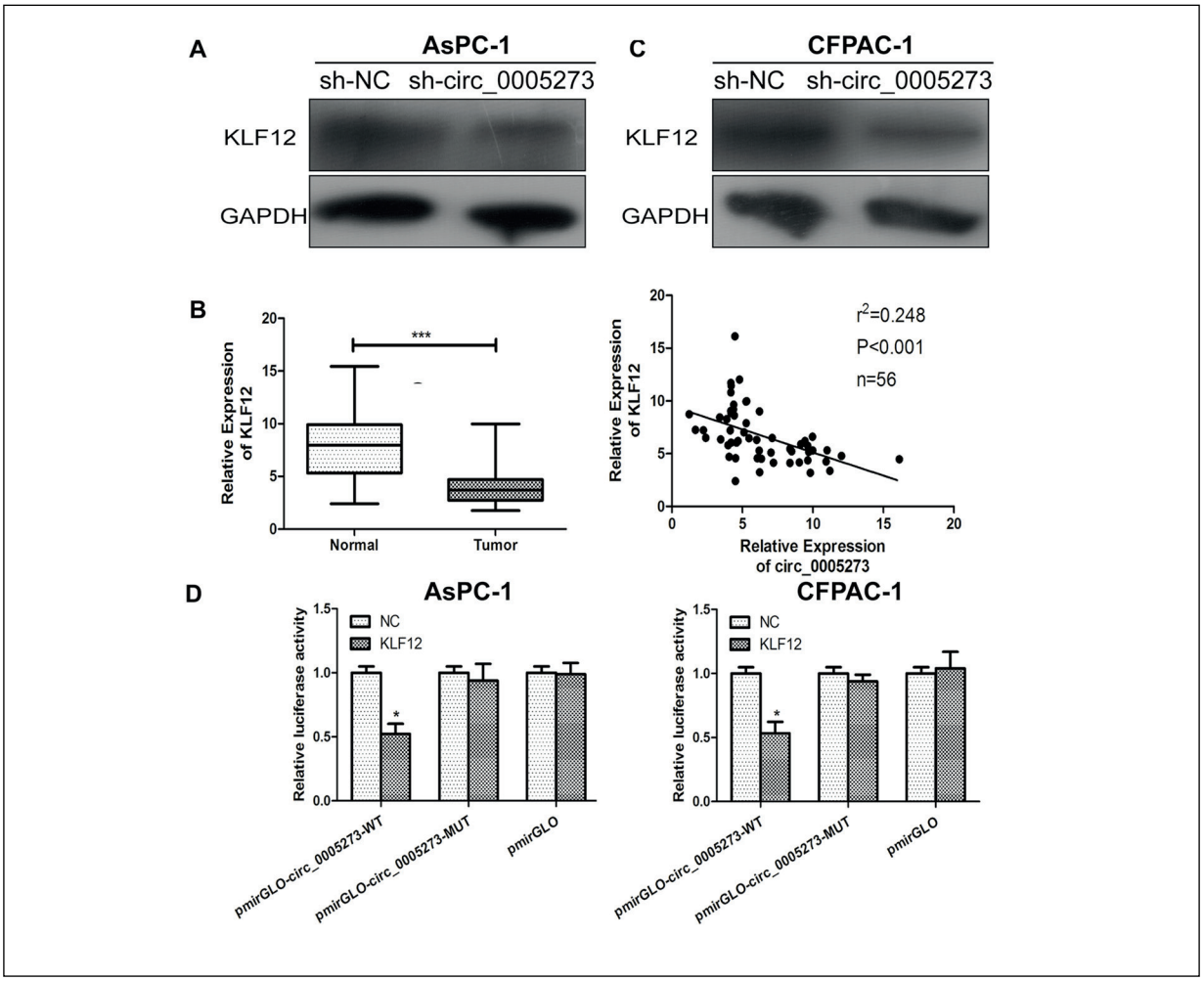

Figure 3. Circ_0005273 bound to KLF12. **A,** Protein levels of KLF12 in AsPC-1 and CFPAC-1 cells transfected with sh-NC or sh-circ_0005273; **B,** Differential expressions of KLF12 in pancreatic cancer tissues and normal tissues; **C,** A negative expression correlation between circ_0005273 and KLF12; **D,** Luciferase activity in AsPC-1 and CFPAC-1 cells. Data were expressed as mean±SD. *$p < 0.05$, ***$p < 0.001$.

circ_0005273-WT. Meanwhile, the mutant sequences were similarly inserted to generate pmirGLO-circ_0005273-MUT. Then, pmirGLO was co-transfected into AsPC-1 and CFPAC-1 cells with either pcDNA-NC or pcDNA-KLF12. It was discovered that Luciferase activity was decreased in cells co-transfected with pmirGLO-circ_0005273-WT and pcDNA-KLF12, confirming the binding between circ_0005273 and KLF12 (Figure 3D).

KLF12 Was Responsible for Circ_0005273-Modulated Phenotypes of Pancreatic Cancer

To further elucidate the role of KLF12 in pancreatic cancer progression, transfection efficacy of si-KLF12 was examined in AsPC-1 and CFPAC-1 cells with circ_0005273 knockdown (Figure 4A). Notably, the decreased migratory cell number and wound closure percentage following knockdown of circ_0005273 were abolished by silenced KLF12 (Figure 4B, 4C).

Discussion

The occurrence and progression of solid tumors involve various biological behaviors, including tumor cell growth and metastasis, drug resistance, tumor relapse, tumor stemness and tumor angiogenesis[22,23]. These malignant phenotypes are closely related to the prognosis of tumor patients[23,24]. Genetic and epigenetic changes vary a lot during tumorigenesis. RNAs and proteins

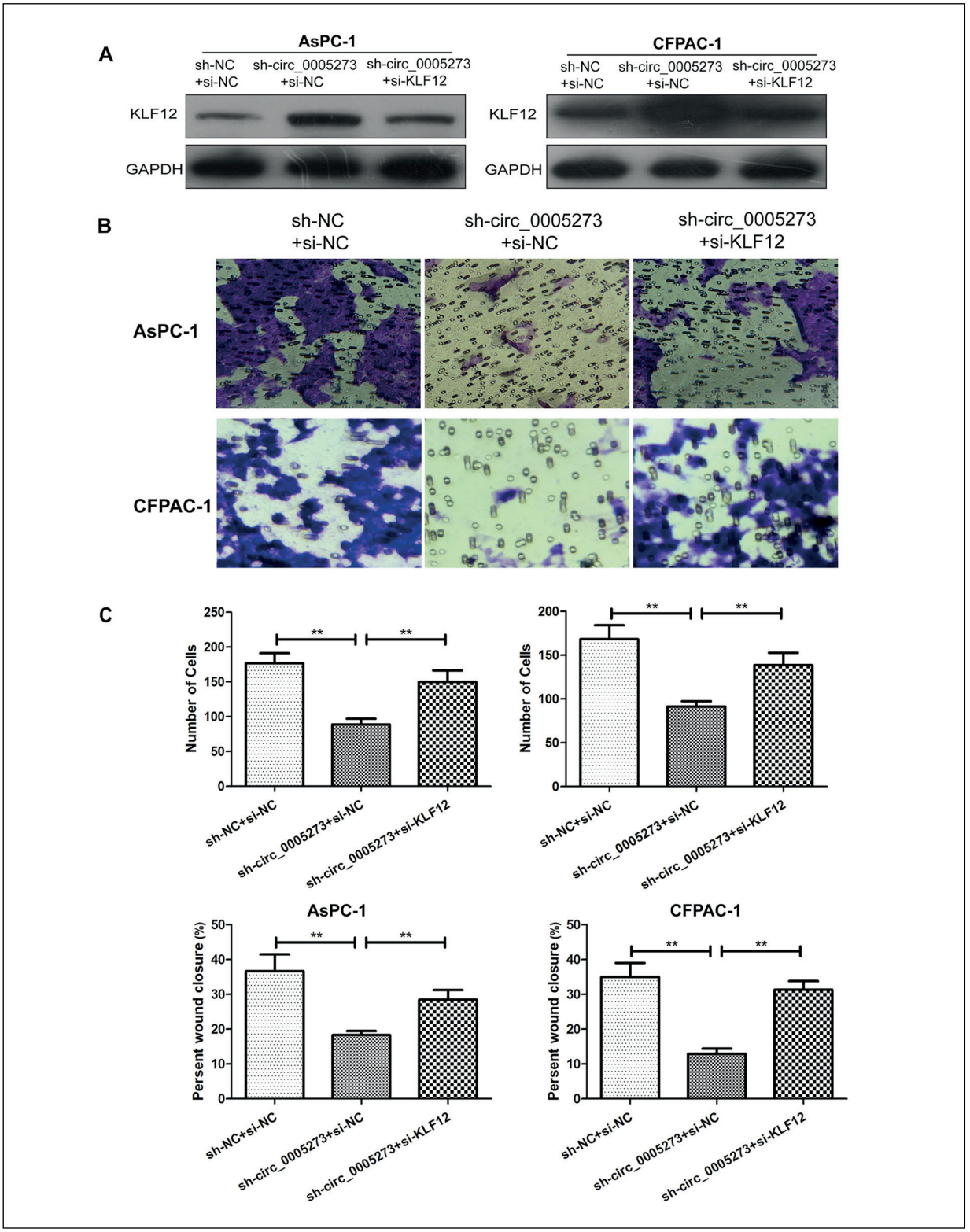

Figure 4. KLF12 was responsible for circ_0005273-modulated phenotypes of pancreatic cancer. **A,** Protein level of KLF12 in AsPC-1 and CFPAC-1 cells co-transfected with sh-NC+si-NC, sh-circ_0005273+si-NC or sh-circ_0005273+si-KLF12; **B,** Migration in AsPC-1 and CFPAC-1 cells co-transfected with sh-NC+si-NC, sh-circ_0005273+si-NC or sh-circ_0005273+si-KLF12 (magnification: 40×); **C,** Wound closure in AsPC-1 and CFPAC-1 cells co-transfected with sh-NC+si-NC, sh-circ_0005273+si-NC or sh-circ_0005273+si-KLF12. Data were expressed as mean±SD. $^{**}p < 0.01$.

are differentially expressed between normal tissues and tumor ones[25,26], and these linked and connected changes ultimately cause cell canceration and tumor progression[26,27].

CircRNAs are highly stable, extensively distributed, evolutionarily conserved and time-specific[12,13]. As circRNAs do not have 5' or 3'ends, they are resistant to enzyme degradation. The stable structure of circRNAs allows them to be important in homeostasis[14-16]. Differing from normal tissues, circRNAs are abnormally expressed in tumor profiling, and these circRNAs can be determinant during tumor progression as oncogenes or tumor suppressors[17-19]. To uncover the potential role of circRNAs in the malignant progression of pancreatic cancer, circ_0005273 was selected as the research object *via* database analysis and literature review[20,21]. However, its biological functions in pancreatic cancer are unclear. The findings of this study showed that circ_0005273 was upregulated in pancreatic cancer tissues compared with that in normal tissues. In addition, its level was linked to lymphatic metastasis, distant metastasis and prognosis in pancreatic cancer patients. Subsequently, a circ_0005273 knockdown model was generated in AsPC-1 and CFPAC-1 cells. Knockdown of circ_0005273 markedly inhibited proliferative and migratory potentials of pancreatic cancer cells. It is suggested that circ_0005273 may be a vital gene used for predicting the progression of malignant progression.

Unlike traditional non-coding RNAs, circRNAs are able to encode proteins or peptides relying on cis-regulatory elements of IRES sequences. The protein-encoding functions of circRNAs initiate from the middle part of circRNA sequences, and the translation rate is slower than the typical one that translates from the start[14-17]. Using bioinformatics tools, the interaction between circ_0005273 and KLF12 was predicted. Knockdown of circ_0005273 markedly downregulated vital genes in the KLF12 in pancreatic cancer genes. KLF12 is a regulator for transcription factors. It is well-known that transcription factors are the terminals of cell signaling pathways. They can integrate upstream abnormal signals and lead to malignant phenotypes or abnormal behaviors of cells through directly regulating tumor-associated genes. Hence, transcription factors exert a direct role in tumor progression[28,29]. KLF12 was downregulated in pancreatic cancer tissues that were collected, showing a negative correlation with circ_0005273 level. The subsequent Dual-Luciferase reporter assay further showed the binding between KLF12 and circ_0005273. Based on their close relationship, it was reckoned that KLF12 may participate in the progression of pancreatic cancer. Of note, the inhibited migratory ability in pancreatic cancer cells with circ_0005273 knockdown was abolished by silenced KLF12. As a result, KLF12 was responsible for the malignant progression of pancreatic cancer regulated by circ_0005273. To sum up, as a novel oncogenic gene, circ_0005273 could aggravate the malignant development of pancreatic cancer. In addition, a negative feedback loop circ_0005273-KLF12 axis has been identified, which becomes a promising biomarker for the diagnosis and treatment of pancreatic cancer.

Conclusions

Circ_0005273 drives proliferative and migratory abilities of pancreatic cancer cells *via* activating the KLF12, and it is able to predict lymphatic metastasis, distant metastasis and prognosis in pancreatic cancer patients.

Conflict of Interest

The Authors declare that they have no conflict of interests.

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