

Up-Regulation of circEIF6 Contributes to Pancreatic Cancer Development Through Targeting miR-557/*SLC7A11*/PI3K/AKT Signaling

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Background: Accruing evidences have pointed out that abnormal expression of circular RNAs (circRNAs) was closely related to the development of many malignancies. The present study intended to disclose the role of circRNA eukaryotic translation initiation factor 6 (circEIF6; hsa_circ_0060055) in pancreatic cancer progression.

Methods: Quantitative real-time polymerase chain reaction (qRT-PCR) was conducted to determine the expression of circEIF6, *EIF6* messenger RNA (mRNA), microRNA-557 (miR-557) and solute carrier family 7 member 11 (*SLC7A11*) mRNA. Cell proliferation ability, migration and invasion abilities and apoptosis were evaluated by Cell Counting Kit 8 (CCK8) assay, transwell migration and invasion assays and flow cytometry. Western blot assay was performed for the expression determination of all proteins. The predicted interaction between miR-557 and circEIF6 or *SLC7A11* was confirmed by dual-luciferase reporter assay. Xenograft tumor model was used for exploring the biological function of circEIF6 in vivo.

Results: CircEIF6 abundance was aberrantly up-regulated in pancreatic tumor tissues and cell lines. Cell proliferation, migration and invasion were significantly restrained while cell apoptosis was induced with the silencing of circEIF6 in pancreatic cancer cells. CircEIF6 silencing also hampered the activation of phosphatidylinositol 3-kinase (PI3K)/AKT serine/threonine kinase (AKT) pathway. CircEIF6 bound to miR-557, and circEIF6 silencing elevated the expression of miR-557 in pancreatic cancer cells. MiR-557 knockdown partly overturned circEIF6 silencing-induced effects in pancreatic cancer cells. *SLC7A11* was a target of miR-557, and miR-557 overexpression suppressed malignant potential of pancreatic cancer cells partly through reducing the expression of *SLC7A11*. CircEIF6 knockdown blocked xenograft tumor growth in vivo.

Conclusion: CircEIF6 aggravated pancreatic cancer development through promoting cell proliferation, migration and invasion and suppressing cell apoptosis through targeting miR-557/*SLC7A11*/PI3K/AKT signaling.

Keywords: pancreatic cancer, EIF6, miR-557, *SLC7A11*, PI3K/AKT signaling

Introduction

Pancreatic cancer is a common digestive tract cancer that ranks the fourth in cancer-associated deaths globally.^{1,2} Most pancreatic cancer patients were diagnosed at an advanced condition at the first visit because of the asymptomatic onset of pancreatic cancer.³ Therefore, understanding the potential mechanism that drives pancreatic cancer development is important for developing effective diagnostic techniques and therapeutic strategies.

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Circular RNAs (circRNAs) are characterized by stable circular structure due to the covalently closed ends.⁴ Recently, circRNAs have become the focus in the fields of human diseases and cancers.^{5,6} Accumulating evidences have shown that circRNAs function as modulators in cancer development, and they may be novel bio-markers and therapeutic targets.^{4,7} CircRNA eukaryotic translation initiation factor 6 (circEIF6; hsa_circ_0060055) was abnormally up-regulated in pancreatic ductal adenocarcinoma tissues compared with adjacent normal tissues.⁸ Nevertheless, the precise contribution of circEIF6 in pancreatic cancer development is largely unknown.

MicroRNAs (miRNAs) are small non-coding RNAs that have been extensively studied in human cancer field.⁹ Many miRNAs serve as oncogenes or tumor suppressors in pancreatic cancer.¹⁰ For instance, miR-9-5p functioned as a tumor suppressor to suppress the malignant behaviors of pancreatic cancer cells through targeting GOT1.¹¹ Zhang et al demonstrated that miR-135b-5p accelerated the aggressive phenotypes of pancreatic cancer cells through targeting NR3C2.¹² MiR-557 overexpression restrained the proliferation capacity and invasion capacity in pancreatic cancer cells through targeting EGFR.¹³ Here, the mechanism of miR-557 in pancreatic cancer progression was further explored.

MiRNAs generally function as regulators in cancer development through targeting messenger RNAs (mRNAs).¹⁴ Solute carrier family 7 member 11 (*SLC7A11*) functions in modulating the transportation of amino acid.^{15,16} Former works have pointed out that the expression of *SLC7A11* was up-regulated in cancers, and a high level of *SLC7A11* was associated with malignant behaviors of cancer cells.^{17,18} Zhu et al claimed that miR-139-5p hampered cellular malignant proliferation and motility of pancreatic cancer cells through targeting *SLC7A11*/phosphatidylinositol 3-kinase (PI3K)/AKT serine/threonine kinase (AKT) signaling.¹⁹ Here, we investigated the potential mechanism behind the oncogenic role of *SLC7A11* in pancreatic cancer.

The current study intended to analyze the expression pattern of circEIF6 in pancreatic cancer and investigate the downstream molecular mechanism by which circEIF6 contributes to pancreatic cancer progression.

Materials and Methods

Clinical Samples

Pancreatic tumor tissues (n=39) along with matched adjacent normal tissues (n=39; at least 2 cm away

from the tumor border) were collected from pancreatic cancer patients at The First People's Hospital of Jingzhou. No subject had received neoadjuvant treatment, and all subjects had signed informed consents before surgical resection. There were 17 patients with pancreatic tumors of I and II phase, while there were 22 patients with pancreatic tumors of III phase according to the Tumor-Node-Metastasis (TNM) clinical staging criteria.²⁰ All protocols were given permission by the ethics committee of The First People's Hospital of Jingzhou and was carried out according to the guidelines of the Declaration of Helsinki.

Cell Lines

Normal human pancreatic duct epithelial cell line (HPDE) and two pancreatic cancer cell lines (Hs 766T and SW1990) were purchased from BeNa Culture Collection (Beijing, People's Republic of China) and cultured with Dulbecco's modified Eagle's medium (DMEM; Gibco, Carlsbad, CA, USA) added with 10% fetal bovine serum (FBS; Gibco) and 1% antibiotic (100 units/mL penicillin/100 µg/mL streptomycin; Solarbio, Beijing, People's Republic of China) in a 37°C incubator with 5% CO₂/95% air.

Cyclization Validation

RNA samples extracted from Hs 766T or SW1990 cells were divided into two equal parts, and 2 µg RNA samples were incubated with 6U RNase R EIF6 and linear *EIF6* mRNA were examined by qRT-PCR.

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

RNA samples from tissues and cells were prepared with Trizol reagent (Invitrogen, Carlsbad, CA, USA). For miR-557 analysis, complementary DNA (cDNA) was synthesized using First-Strand cDNA Synthesis Kit (Takara, Dalian, People's Republic of China), and PCR was conducted using SYBR Premix Ex TaqTM EIF6, *EIF6* mRNA and *SLC7A11* mRNA, RNA was converted into cDNA with One Step TB GreenTM PrimeScriptTM RT-PCR Kit (Bio-Rad, Hercules, CA, USA) followed by PCR reaction using iQ SYBR Green Supermix (Bio-Rad). The specific primer sequences are shown in Table 1. Glyceraldehyde-3-EIF6, *EIF6* mRNA and *SLC7A11* mRNA, while U6 acted as the control for

Table 1 Primers Used in qRT-PCR

Gene	Species	Direction (5'-3')	Sequence
EIF6	Human	Forward Reverse	TGCAAAAACCTGCTCTGTTG CTGTTCCCCACACACATGC
EIF6	Human	Forward Reverse	AGAACTTCTACAGTGTGTTT CATTGCAGGTGGTGACATTG
miR-557	Human	Forward Reverse	ACACTCCAGCTGGGGTTTGCACGGGTGGGC TGGTGTCTGGAGTCC
SLC7A11	Human	Forward Reverse	GCTGTGATATCCCTGGCATT GGCGTCTTAAAGTTCTGCG
U6	Human	Forward Reverse	CTCGCTTCGGCAGCAC AACGCTTCACGAATTTGCGT
GAPDH	Human	Forward Reverse	ACAACTTTGGTATCGTGAAGG GCCATCACGCCACAGTTTC

miR-557. The $2^{-\Delta\Delta Ct}$ method was used for the quantification of these molecules.

Cell Transfection

CircEIF6 small interfering RNAs (siRNAs), including si-circEIF6#1 (5'-UCGAACACACCGGAGGGAACU-3'), si-circEIF6#2 (5'-AACACACCGGAGGGAACUGCA-3') and si-circEIF6#3 (5'-ACACACCGGAGGGAACUGCAC-3'), negative control siRNA (si-NC), circEIF6 short hairpin RNA (sh-circEIF6), sh-NC, miR-557 mimics (miR-557), miR-NC, miR-557 inhibitor (in-miR-557) and in-miR-NC were purchased from GenePharma (Shanghai, People's Republic of China). For the overexpression of *SLC7A11*, we introduced the full length of *SLC7A11* into pcDNA vector to obtain *SLC7A11* recombinant overexpression plasmid, and pcDNA empty vector was used as the control. Lipofectamine 3000 reagent (Invitrogen) was used to transfect these RNA and plasmid into Hs 766T or SW1990 cells.

Cell Counting Kit 8 (CCK8) Assay

Hs 766T or SW1990 cells were digested and seeded into 96-well plates. After a fixed time interval after transfection, pancreatic cancer cells were incubated with CCK8 reagent (20 μ L; Dojindo Laboratories, Kumamoto, Japan) for 1 hour. The absorbance at 450 nm was measured by the microplate reader (Nikon, Tokyo, Japan).

Transwell Migration and Invasion Assays

A total of 40 μ L Matrigel (BD biosciences, San Jose, CA, USA) at the dilution of 1:8 was added to the transwell upper

chambers for 30 minutes at 37°C for solidification to conduct transwell invasion assay, while un-coated transwell chambers were used for transwell migration assay. After transfection for 24 hours, pancreatic cancer cells in serum-free medium were seeded into the upside of the membrane, while the lower chambers were filled with medium added with 10% FBS. Transwell plates were placed in a cell incubator for 24 hours. Cells migrated or invaded to the lower surface of the membrane were washed with phosphate buffer saline (PBS, Sangon Biotech, Shanghai, People's Republic of China), fixed with 4% paraformaldehyde (Sigma, St. Louis, MO, USA) and stained with 0.5% crystal violet (Sangon Biotech). The numbers of migrated and invaded cells in five random fields were counted at 100 \times magnification. The scale bar indicates 200 μ M.

Flow Cytometry

After transfection for 72 hours, the DNA content and phosphatidylserine in transfected Hs 766T or SW1990 cells were stained using propidium iodide (PI; R&D systems, Abingdon, UK) and fluorescein isothiocyanate (FITC)-labeled Annexin V (Annexin V-FITC; R&D systems) for 15 minutes in the dark, respectively. The proportion of apoptotic cells (the first quadrant and the fourth quadrant) was analyzed by FC-500 flow cytometer (Beckman Coulter, Pasadena, CA, USA).

Western Blot Assay

After transfection for 24 hours, Hs 766T or SW1990 cells were lysed by Western cell lysis buffer (Beyotime, Haimen,

People's Republic of China). An equal amount of protein samples was loaded onto sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) gel and blotted onto polyvinylidene fluoride (PVDF) membrane (Millipore, Billerica, MA, USA). The membrane was incubated with 5% skimmed milk for 1 hour at room temperature for blocking. Primary antibodies against p-AKT (07-1398; Sigma), AKT (SAB4500797; Sigma), p-PI3K (ab389562; Abcam, Cambridge, MA, USA), PI3K (ab32089; Abcam), *SLC7A11* (SAB2500951; Sigma) and β -actin (A1978; Abcam) were incubated with the membrane at 4°C overnight. Afterwards, horseradish-peroxidase (HRP)-labeled secondary antibody (Sigma) was incubated with the membrane for 2 hours at room temperature. Immunochemical detection was conducted with the enhanced chemiluminescence (ECL) Detection System (GE Healthcare, Chicago, IL, USA).

Bioinformatic Prediction

Circular RNA Interactome software (<https://circinteractome.nia.nih.gov>) was used to predict circEIF6-miRNA interactions, while the mRNA targets of miR-557 were predicted by TargetScan software (<http://www.targets.can.org>).

Dual-Luciferase Reporter Assay

CircEIF6 or *SLC7A11* 3' untranslated region (3'UTR) sequence, containing the wild-type (WT) miR-557 binding sites, was fused to the luciferase reporter vector pmirGLO vector (Promega, Madison, WI, USA), termed as circEIF6 WT and *SLC7A11* 3'UTR WT. Also, site-directed mutagenesis in miR-557-binding sites in circEIF6 or *SLC7A11* 3'UTR sequence was made to generate circEIF6 MUT and *SLC7A11* 3'UTR MUT. Hs 766T or SW1990 cells were co-transfected with miR-NC or miR-557 and luciferase plasmids with Lipofectamine 3000 reagent (Invitrogen), and the luciferase activity was determined by the Dual-Luciferase Reporter System Kit (Promega) after transfection for 48 hours.

Xenograft Tumor Model

Animal experiments were conducted with the permission of the animal care and use committee of The First People's Hospital of Jingzhou and performed in accordance with the guidelines of the National Animal Care and Ethics Institution. SW1990 cell line stably transfected with sh-EIF6 or sh-NC was established. Afterwards, SW1990 cells stably transfected with sh-EIF6 or sh-NC were subcutaneously inoculated into the right flank of BALB/c male

nude mice (Vital River Laboratory Animal Technology, Beijing, People's Republic of China) in sh-NC group and sh-EIF6 group. The volume of tumors was recorded every week using the caliper as $\text{width}^2 \times \text{length} \times 0.5$. After inoculation for 28 days, these nude mice were euthanized and the tumors were dissected and weighed. The levels of circEIF6 and miR-557 were detected by qRT-PCR, while Western blot assay was conducted to examine the levels of *SLC7A11*, p-AKT, AKT, p-PI3K and PI3K.

Statistical Analysis

The statistical results are represented as mean \pm standard deviation. Each experiment was repeated for three times. Statistical analysis was conducted by Student's *t*-test (in two groups) or one-way analysis of variance (ANOVA) followed by Tukey's test (in more than two groups) using GraphPad Prism 7.0 software. Linear correlation among circEIF6, miR-557 and *SLC7A11* was assessed by Pearson correlation coefficient. Difference was defined as statistically significant with the *P* value of less than 0.05.

Results

CircEIF6 is Highly Expressed in Pancreatic Cancer Tissues and Cell Lines

CircEIF6 expression was significantly enhanced in pancreatic tumor tissues relative to adjacent normal tissues (Figure 1A). Pancreatic cancer patients were grouped into I+II group (n=17) and III group (n=22) on the basis of TNM staging. As displayed in Figure 1B, circEIF6 expression was observed to be notably enhanced in tumor tissues of advanced stage (III stage) relative to that in I+II stage, suggesting that circEIF6 expression was positively related to the malignant grade of pancreatic cancer. ROC curve was generated to assess the diagnostic value of circEIF6 expression in tumor tissues of pancreatic cancer patients. The ROC curve revealed an area under the curve (AUC) of 0.9093 (95% confidence interval (CI)=0.845–0.9735) (Figure 1C). Furthermore, a significant elevation in circEIF6 expression was discovered in two pancreatic cancer cell lines relative to HPDE cell line (Figure 1D). RNase R was used to evaluate the circular characteristics of circEIF6. RNA samples from Hs 766T and SW1990 cells were treated with RNase R or not, and we found that the level of linearEIF6 rather than circEIF6 was dramatically reduced upon RNase R digestion (Figure 1E and F), providing strong evidence that circEIF6 possessed circular structure.

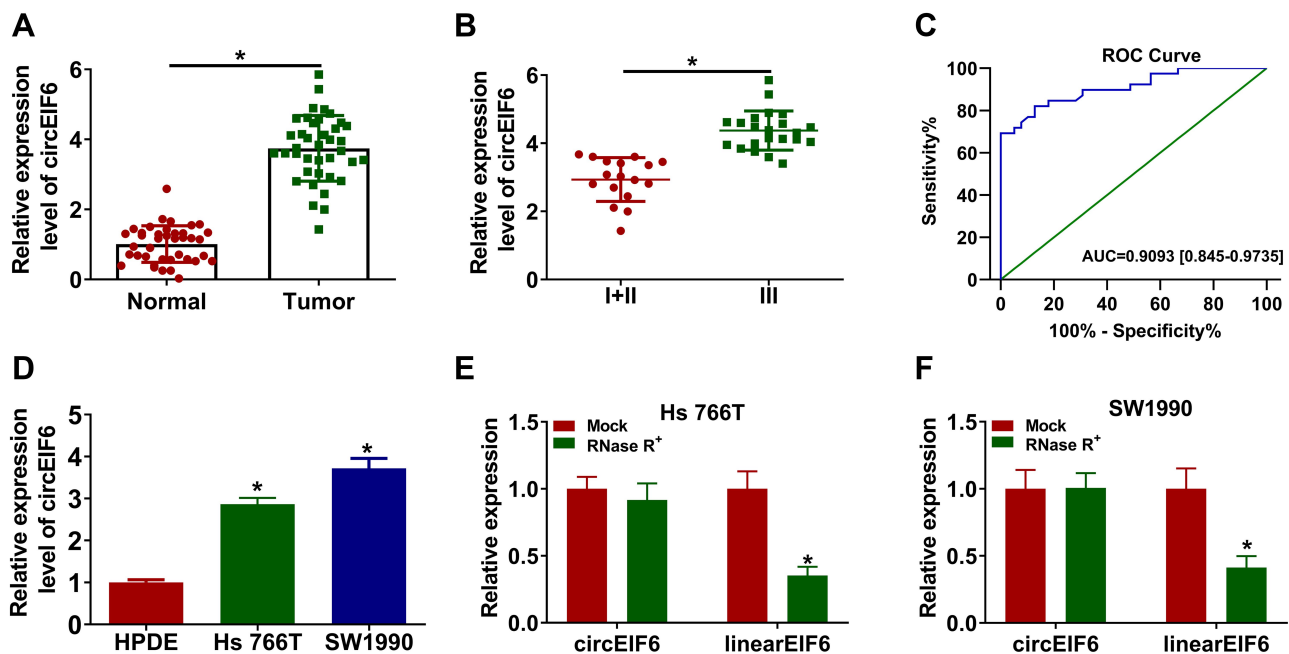


Figure 1 CircEIF6 is highly expressed in pancreatic cancer tissues and cell lines. **(A)** The level of circEIF6 in tumor tissues (n=39) and adjacent normal tissues (n=39) from pancreatic cancer patients was evaluated by qRT-PCR. **(B)** The expression of circEIF6 in the tumor tissues from pancreatic cancer patients in I+II phase and III phase was assessed by qRT-PCR. **(C)** ROC curve was used to analyze the diagnostic value of circEIF6 in pancreatic cancer patients. **(D)** qRT-PCR was used to measure the expression of circEIF6 in normal human pancreatic duct epithelial cell line (HPDE) and two pancreatic cancer cell lines (Hs 766T and SW1990). **(E and F)** The stability of circEIF6 along with its linear form EIF6 mRNA was assessed using RNase R by qRT-PCR. *P<0.05.

Overall, abnormal expression of circEIF6 might imply its important function in pancreatic cancer progression.

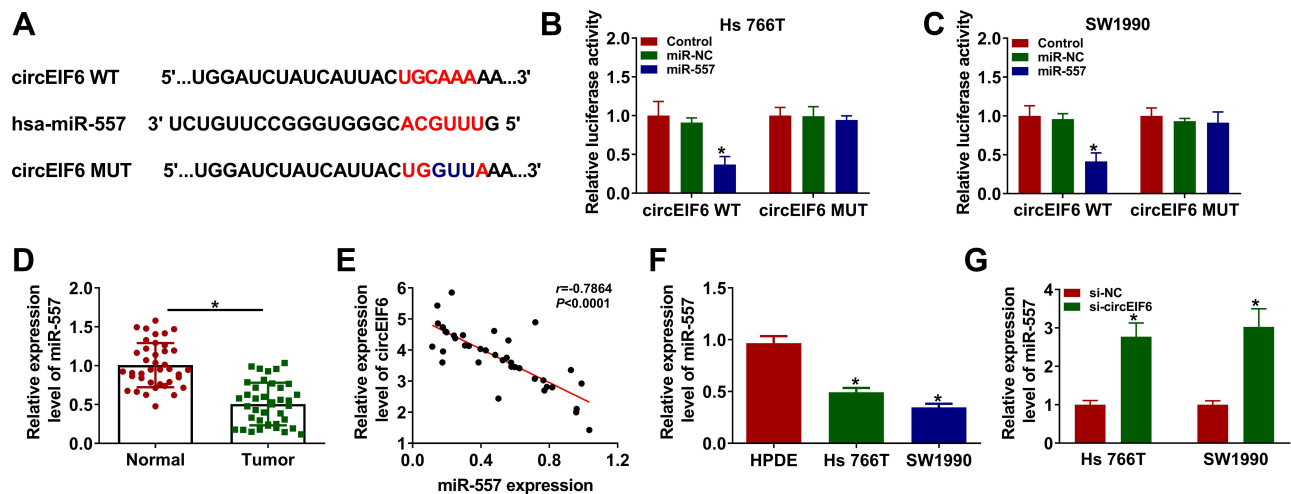
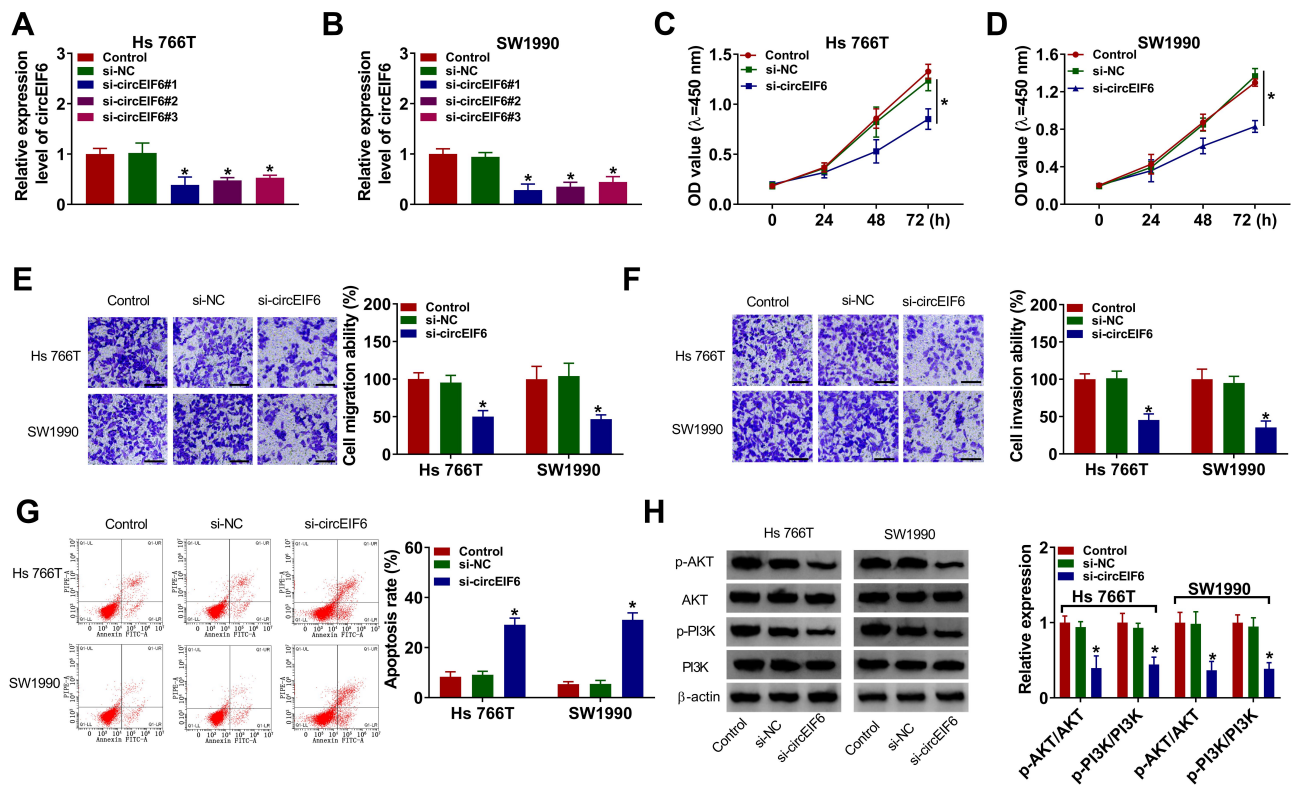
CircEIF6 Knockdown Hampers Cell Proliferation, Migration, Invasion and Induces Cell Apoptosis of Pancreatic Cancer Cells

To characterize the function of circEIF6 in pancreatic cancer, we designed three siRNAs targeting circEIF6 to silence its expression in pancreatic cancer cells. As shown in [Figure 2A and B](#), among three siRNAs of circEIF6, si-circEIF6#1 (written as si-EIF6 in the following experiments) was selected for the following experiments due to its the highest transfection efficiency. As examined by CCK8 assay, cell proliferation was significantly suppressed with the silencing of circEIF6 compared with si-NC-transfected pancreatic cancer cells ([Figure 2C and D](#)). As mentioned in [Figure 2E and F](#), cell migration ability and invasion ability were both significantly restrained following the transfection of si-EIF6 compared with si-NC group. As determined by flow cytometry, cell apoptosis was triggered following the transfection of si-EIF6 than that in si-NC group ([Figure 2G](#)). The activity of PI3K/AKT signaling was analyzed through measuring the levels of p-AKT, AKT,

p-PI3K and PI3K in pancreatic cancer cells. As displayed in [Figure 2H](#), after silencing circEIF6, the levels of p-AKT/AKT and p-PI3K/PI3K were both reduced than that in si-NC group in the two pancreatic cancer cell lines. Taken together, circEIF6 silencing blocked cell proliferation, migration, invasion and promoted cell apoptosis of pancreatic cancer cells.

MiR-557 is a Target of circEIF6 in Pancreatic Cancer Cells

Bioinformatic search using Circular RNA Interactome was conducted to identify the possible miRNA targets of circEIF6. Among all the candidate miRNA targets (Circular RNA Interactome software) of circEIF6, only miR-557 has been reported to be down-regulated in pancreatic cancer,¹³ thus we performed dual-luciferase reporter assay to test the target interaction between miR-557 and circEIF6. The complementary sequence between circEIF6 and miR-557 is shown in [Figure 3A](#). Dual-luciferase reporter assay was performed in pancreatic cancer cells to further verify the target interaction between miR-557 and circEIF6. Transfection with miR-557 rather than miR-NC significantly reduced the luciferase intensity of luciferase reporter circEIF6 WT in Hs 766T and SW1990 cells ([Figure 3B and C](#)). Furthermore, luciferase activities were unchanged with the transfection of reporter



circEIF6 MUT upon the co-transfection of miR-NC or miR-557 (Figure 3B and C), providing evidence that miR-557 was a target of circEIF6 in pancreatic cancer cells. MiR-557 expression was significantly decreased in pancreatic tumor tissues when compared with adjacent normal tissues (Figure 3D). An obvious negative correlation was observed between the expression of circEIF6 and miR-557 in pancreatic tumor tissues (Figure 3E). Compared with HPDE cell line, miR-557 expression was notably reduced in Hs 766T and SW1990 cells (Figure 3F). CircEIF6 silencing caused a significant up-regulation in miR-557 expression in both the two pancreatic cancer cell lines (Figure 3G). Taken together, circEIF6 interacted with miR-557 in pancreatic cancer cells.

CircEIF6 Knockdown Restrains the Malignant Phenotypes of Pancreatic Cancer Cells Through Enhancing miR-557 Level

To explore if circEIF6-induced influences in pancreatic cancer cells required the involvement of miR-557, we co-transfected Hs 766T and SW1990 cells with si-NC, si-circEIF6, si-EIF6 +

in-miR-NC or si-EIF6 + in-miR-557. As displayed in Figure 4A, circEIF6 knockdown-mediated up-regulation of miR-557 level was largely mitigated by the addition of miR-557 inhibitor (in-miR-557). CircEIF6 interference suppressed cell proliferation as evidenced by the cell proliferation curve shown in Figure 4B and C, and miR-557 silencing partly rescued the proliferation ability of pancreatic cancer cells. The migration and invasion abilities were restrained by the silencing of circEIF6, while these effects were largely attenuated by the introduction of in-miR-557 (Figure 4D and E). CircEIF6 interference triggered the apoptosis of pancreatic cancer cells, which was suppressed by the addition of in-miR-557 (Figure 4F). The levels of p-AKT/AKT and p-PI3K/PI3K were reduced with the knockdown of circEIF6, while these influences were largely counteracted by the silencing of miR-557 (Figure 4G and H), suggesting that circEIF6 interference suppressed the activity of PI3K/AKT pathway partly through up-regulating miR-557. These results together demonstrated that miR-557 silencing attenuated circEIF6 knockdown-mediated effects in the biological behaviors of pancreatic cancer cells.

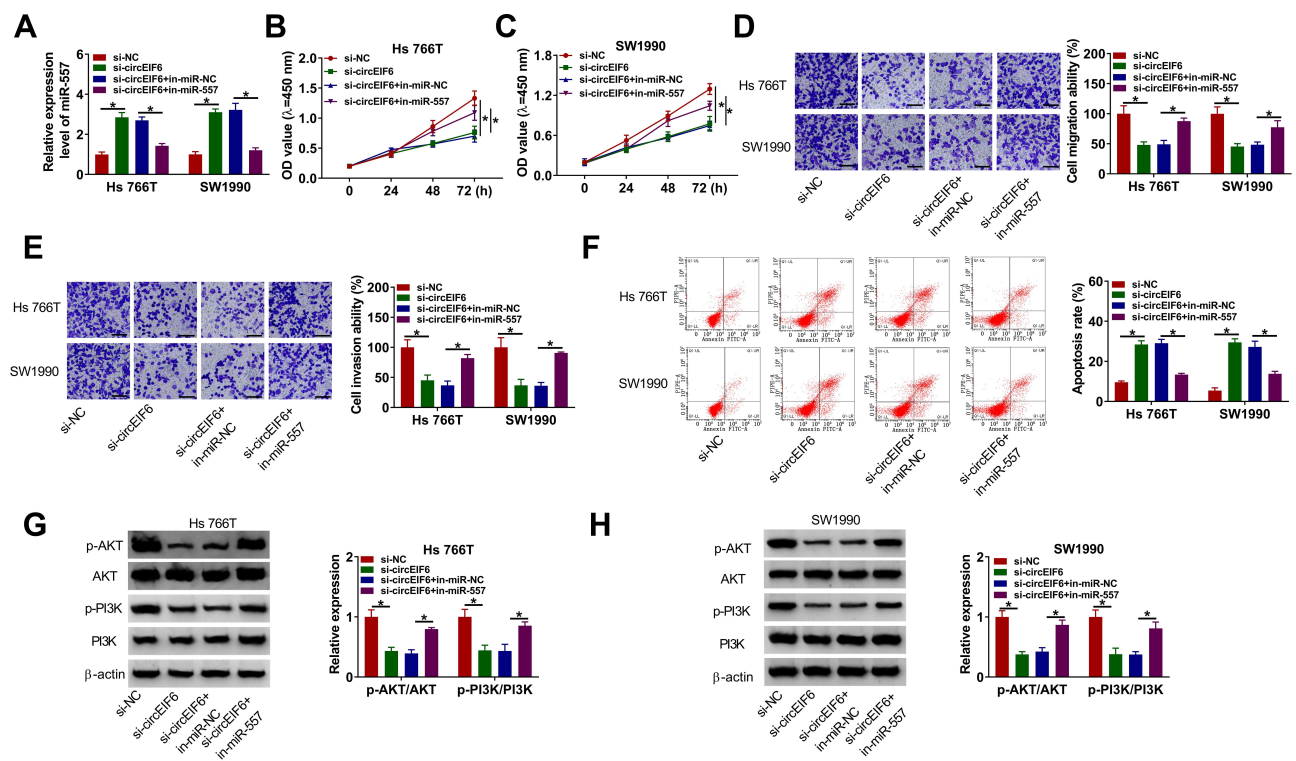


Figure 4 CircEIF6 knockdown restrains the malignant phenotypes of pancreatic cancer cells through enhancing miR-557 level. (A–H) Hs 766T and SW1990 cells were transfected with si-EIF6 alone or together with in-miR-557. (A) Evaluation of miR-557 abundance in transfected pancreatic cancer cells was conducted via qRT-PCR. (B and C) The influences of circEIF6 silencing and miR-557 silencing on the proliferation of pancreatic cancer cells were analyzed by CCK8 assay. (D and E) Transwell assays were implemented to analyze cell migration and invasion abilities in transfected pancreatic cancer cells. (F) Flow cytometry was conducted to count the percentage of apoptotic pancreatic cancer cells (the first quadrant and the fourth quadrant). (G and H) Western blot assay was conducted to detect the abundance of p-AKT, AKT, p-PI3K and PI3K in transfected pancreatic cancer cells. * $P < 0.05$.

SLC7A11 is a Target of miR-557 in Pancreatic Cancer Cells

Bioinformatic software (TargetScan) was used to investigate miR-557-mRNAs interactions. Among all the predicted mRNA targets of miR-557, we screened five mRNA targets that have been reported to be up-regulated in pancreatic cancer, including HMGB2,²¹ SOX2,²² FOXM1,²³ MACC1²⁴ and *SLC7A11*¹⁹ (Supplementary Figure 1A). The regulatory relationships between miR-557 and these five candidate targets in pancreatic cancer cells were tested by qRT-PCR. As shown in Supplementary Figure 1B and 1C, miR-557 overexpression reduced the expression of all these five candidate mRNA targets, and *SLC7A11* was selected for the following experiments due to its the highest obvious negative regulatory relationship with miR-557. The predicted complementary sites between *SLC7A11* 3'UTR and miR-557 are shown in Figure 5A. Three bases "CAA" in the

complementary sites of *SLC7A11* were mutated by "GUU" to test if *SLC7A11* interacted with miR-557 via this predicted sequence. Transfection of miR-557 significantly reduced the luciferase activity with the co-transfection of *SLC7A11* 3'UTR WT reporter plasmid compared with miR-NC and *SLC7A11* 3'UTR WT group (Figure 5B and C). Moreover, transfection with miR-557 or miR-NC in *SLC7A11* 3'UTR MUT group caused indiscriminate luciferase activity (Figure 5B and C), suggesting that *SLC7A11* interacted with miR-557 via "UGCAAA" sequence. The expression characteristics of *SLC7A11* were subsequently explored. *SLC7A11* mRNA and protein expression was enhanced in pancreatic tumor tissues compared with that in adjacent normal tissues (Figure 5D and E). The level of *SLC7A11* protein was notably enhanced in two pancreatic cancer cell lines relative to that in HPDE cell line (Figure 5F). Linear correlation between the expression of *SLC7A11* and miR-557 or circEIF6 was

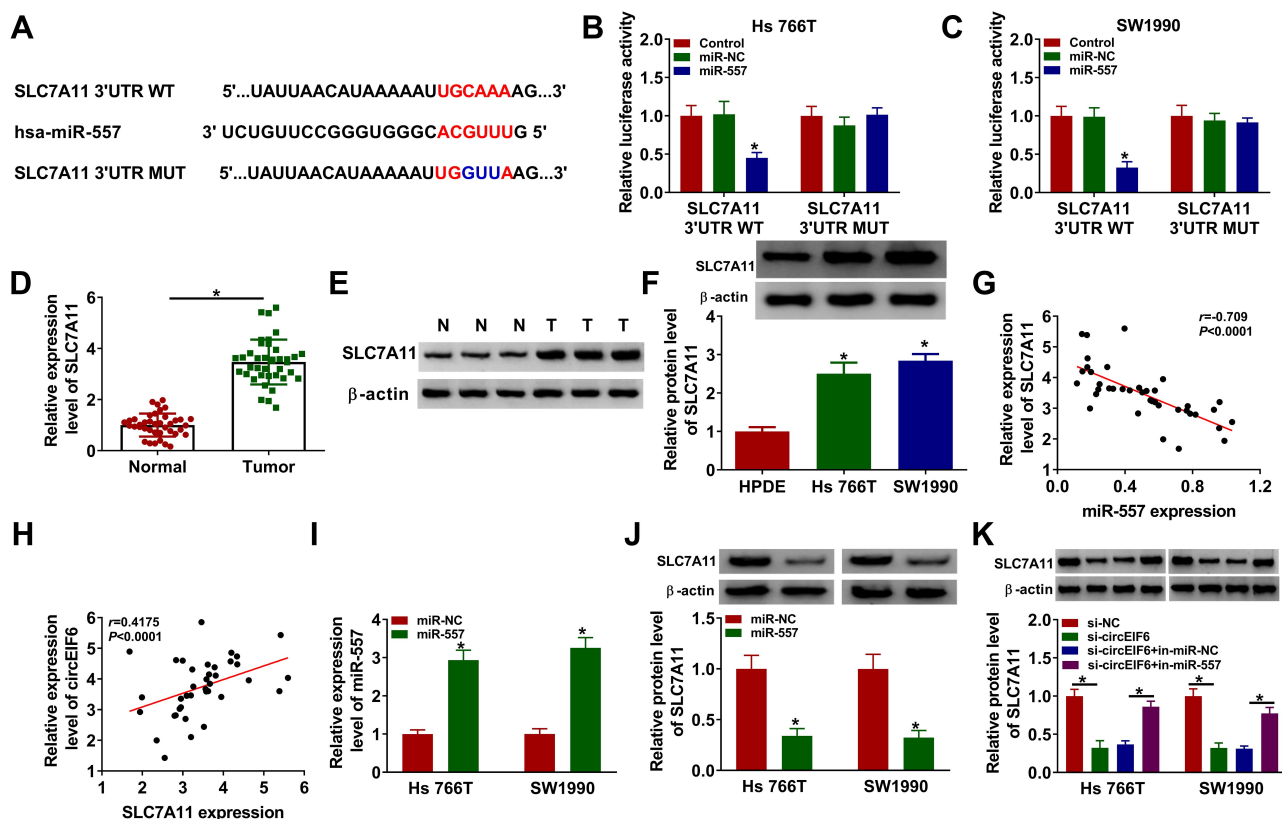


Figure 5 *SLC7A11* is a target of miR-557 in pancreatic cancer cells. (A) The interacted mRNAs of miR-557 were predicted through using bioinformatic software (TargetScan), and *SLC7A11* was predicted to interact with miR-557 via its "UGCAAA" sequence. (B and C) The target interaction between miR-557 and *SLC7A11* was verified by dual-luciferase reporter assay. (D and E) qRT-PCR and Western blot assay were conducted to determine the mRNA and protein expression of *SLC7A11* in pancreatic tumor tissues and adjacent normal tissues. (F) Evaluation of *SLC7A11* level in HPDE, Hs 766T and SW1990 was conducted by Western blot assay. (G and H) The linear relationship between *SLC7A11* and miR-557 or circEIF6 was analyzed by Pearson correlation coefficient. (I) Hs 766T and SW1990 cells were transfected with miR-NC or miR-557, and the level of miR-557 was assessed by qRT-PCR after transfection for 24 hours. (J) Western blot assay was used to analyze the protein expression of *SLC7A11* in Hs 766T and SW1990 cells transfected with miR-NC or miR-557. (K) Evaluation of *SLC7A11* protein expression in pancreatic cancer cells transfected with si-NC, si-circEIF6, si-circEIF6 + in-miR-NC or si-circEIF6 + in-miR-557 was conducted by Western blot assay. * $P < 0.05$.

analyzed by Pearson correlation coefficient. As displayed in Figure 5G and H, *SLC7A11* expression was negatively correlated with the expression of miR-557, while there was a positive correlation between the expression of *SLC7A11* and circEIF6. The overexpression efficiency of miR-557 was assessed by qRT-PCR. The expression of miR-557 was significantly enhanced in pancreatic cancer cells following transfection with miR-557 mimics rather than miR-NC (Figure 5I). After overexpressing miR-557, *SLC7A11* expression was significantly decreased in both Hs 766T and SW1990 cells (Figure 5J). Hs 766T and SW1990 cells were transfected with si-EIF6 alone or together with in-miR-557 to explore the regulatory relationship between circEIF6 and *SLC7A11*. As mentioned in Figure 5K, circEIF6 silencing reduced the protein expression of *SLC7A11*, while this suppressive effect induced by circEIF6 knockdown was largely alleviated by the addition of in-miR-557. Taken together, circEIF6 silencing reduced *SLC7A11* expression through enhancing miR-557 level in pancreatic cancer cells.

***SLC7A11* Overexpression Partly Alleviates miR-557-Mediated Effects in Pancreatic Cancer Cells**

Hs 766T and SW1990 cells were transfected with miR-557 alone or together with *SLC7A11* plasmid to conduct rescue experiments to explore if miR-557-mediated effects were based on its negative regulatory relationship with *SLC7A11*. MiR-557 overexpression down-regulated the expression of *SLC7A11*, while the level of *SLC7A11* was largely rescued with the addition of *SLC7A11* overexpression plasmid (Figure 6A). MiR-557 overexpression resulted in significant suppression in the abilities of proliferation, migration and invasion in pancreatic cancer cells, while these suppressive effects were all attenuated by the addition of *SLC7A11* plasmid (Figure 6B–E). Cell apoptosis was triggered with the forced expression of miR-557, and the co-transfection of miR-557 and *SLC7A11* alleviated the apoptosis of pancreatic cancer cells (Figure 6F). MiR-557 overexpression down-regulated the phosphorylation levels of AKT and PI3K, while the addition of *SLC7A11* plasmid largely recovered the levels of p-AKT/AKT and p-PI3K/PI3K in pancreatic cancer cells (Figure 6G and H). Overall, miR-557 suppressed the malignant potential and the activity of PI3K/AKT signaling through targeting *SLC7A11*.

CircEIF6 Interference Suppresses the Growth of Pancreatic Cancer Xenograft Tumors in vivo

Given the pro-tumor role of circEIF6 in pancreatic cancer in vitro, we subsequently explored the function of circEIF6 in tumor growth in vivo. SW1990 cells stably expressing sh-EIF6 or sh-NC were inoculated into the nude mice. The volume of tumors from sh-EIF6-expressing cells was suppressed compared to those from the sh-NC group (Figure 7A). Also, tumor weight was notably reduced with the interference of circEIF6 compared with the sh-NC group (Figure 7B). The expression of circEIF6 and the protein level of *SLC7A11* were reduced in tumor tissues from sh-EIF6-expressing SW1990 cells compared to those from the sh-NC-expressing SW1990 cells (Figure 7C and E). An opposite tendency in miR-557 expression was found in tumor tissues when compared with the expression trend of circEIF6 or *SLC7A11* (Figure 7D). The levels of p-AKT/AKT and p-PI3K/PI3K were both reduced in the sh-EIF6 group compared with that in the sh-NC group (Figure 7F). Overall, circEIF6 silencing suppressed pancreatic cancer progression in vivo.

Discussion

The discovery of circRNAs provides a novel insight into cancer treatment, which is of vital significance for identifying new diagnostic and therapeutic targets for cancers.^{25,26} Many circRNAs were dysregulated in diverse malignancies, and the clinical significance of dysregulated circRNAs in cancers has been investigated by former works. For instance, circ-LDLRAD3 was highly expressed in the serum samples and tissue samples of pancreatic cancer patients, and high level of circ-LDLRAD3 was related to aggressive phenotype in pancreatic cancer patients.²⁷ Bi et al claimed that circ-ZKSCAN1 expression was reduced in bladder cancer tissues and cell lines, and low expression of circ-ZKSCAN1 was closely associated with the malignant characteristics in bladder cancer patients.²⁸ CircEIF6 exhibited higher expression in pancreatic ductal adenocarcinoma tissue specimens than that in adjacent normal tissue specimens.⁸ However, the biological significance behind the up-regulation of circEIF6 in pancreatic cancer remains to be uncovered. In line with a previous article,⁸ we found that circEIF6 expression was elevated in pancreatic tumor tissues and cell lines. Furthermore, patients with pancreatic cancer in III phase

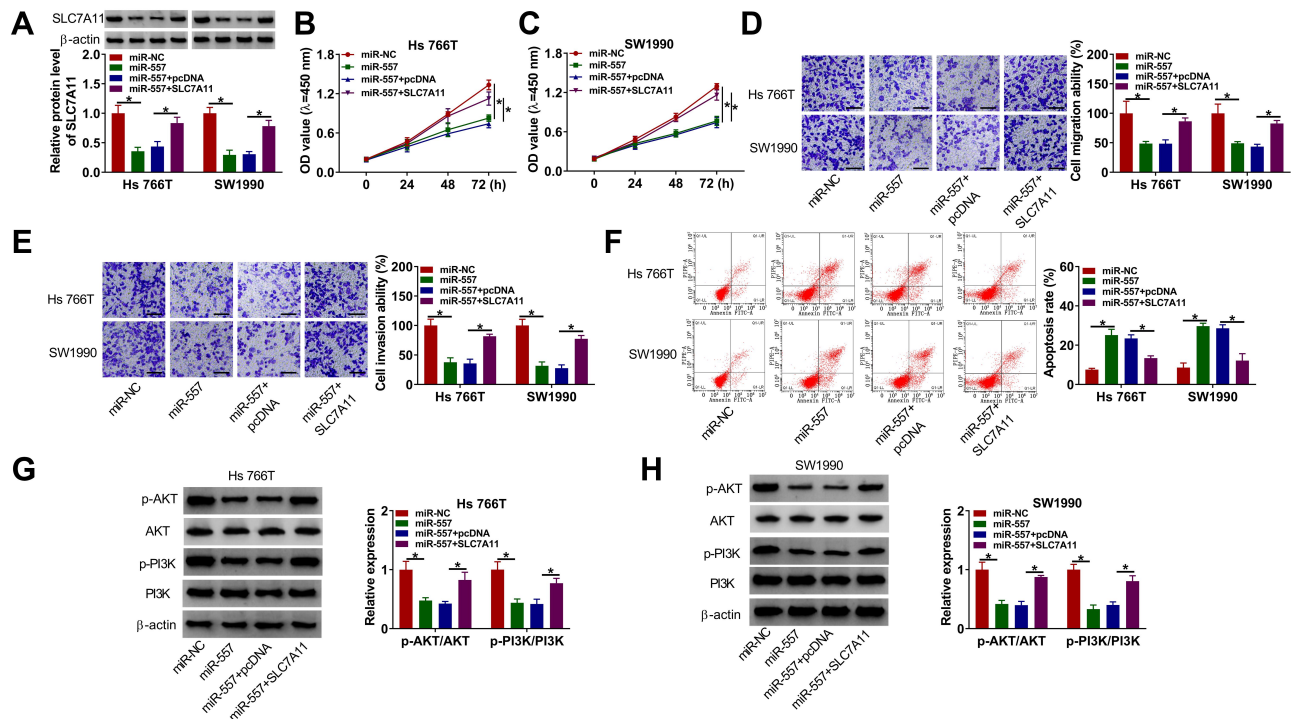


Figure 6 *SLC7A11* overexpression partly alleviates miR-557-mediated effects in pancreatic cancer cells. (A-H) Hs 766T and SW1990 cells were transfected with miR-NC, miR-557, miR-557 + pcDNA or miR-557 + *SLC7A11*. (A) The protein level of *SLC7A11* in transfected pancreatic cancer cells was examined by Western blot assay. (B and C) CCK8 assay was utilized to evaluate cell proliferation ability. (D and E) Transwell assays were conducted to assess cell migration and invasion abilities. (F) The percentage of apoptotic cells (the first quadrant and the fourth quadrant) was evaluated by flow cytometry. (G and H) The levels of p-AKT, AKT, p-PI3K, PI3K were determined by Western blot assay. * $P < 0.05$.

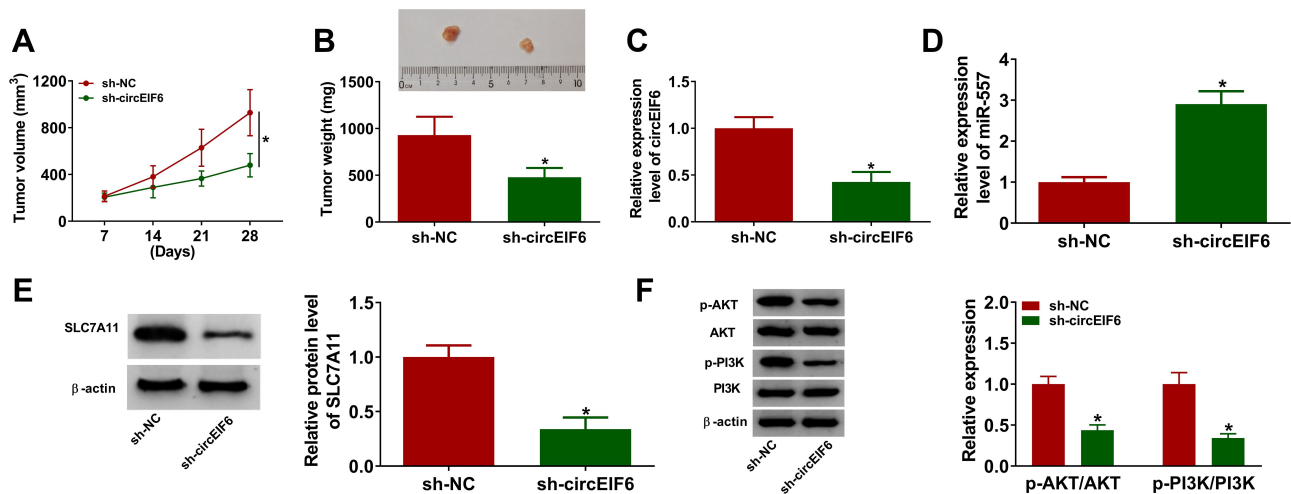


Figure 7 CircEIF6 interference suppresses the growth of pancreatic cancer xenograft tumors in vivo. (A) Tumor volume was calculated every 7 days according to the formula of length \times width \times 0.5. (B) Tumors were weighed after euthanizing mice at 28-day post-inoculation. (C) The expression of circEIF6 in tumor tissues from SW1990 cells transfected with sh-NC or sh-EIF6 was detected by qRT-PCR. (D) The level of miR-557 was measured by qRT-PCR. (E) Western blot assay was used to analyze the protein level of *SLC7A11* in tumor tissues. (F) The levels of p-AKT, AKT, p-PI3K and PI3K were determined by Western blot assay. * $P < 0.05$.

had higher expression of circEIF6 compared with that in I +II phase. CircEIF6 silencing hampered the proliferation, migration and invasion and promoted the apoptosis of pancreatic cancer cells. PI3K/AKT signaling played important roles in suppressing cell apoptosis and inducing

cell proliferation, and abnormal activation of this signaling has been proved to accelerate the progression of many malignancies.^{29,30} For instance, circ_001569 was proved to be an unfavorable prognostic factor to accelerate the proliferation and motility of breast cancer cells through

activating PI3K/AKT signaling.³¹ The forced expression of circ_0000520 attenuated the herceptin resistance of gastric cancer cells through suppressing PI3K/AKT signaling.³² We found that circEIF6 interference also restrained the activity of PI3K/AKT signaling in pancreatic cancer cells.

Through performing bioinformatic analysis and dual-luciferase reporter assay, miR-557 was verified as a target of circEIF6 in pancreatic cancer cells. MiR-557 has been reported to restrain the development of pancreatic cancer and lung cancer. For example, Yang et al demonstrated that miR-557 overexpression suppressed cell proliferation ability and invasion ability in pancreatic cancer cells through targeting EGFR.¹³ Qiu et al claimed that miR-557 suppressed lung cancer development through reducing LEF1 levels.³³ MiR-557 expression was reduced in pancreatic tumor tissues and cell lines, and it was reversely modulated by circEIF6 in pancreatic cancer cells. When we simultaneously transfected si-EIF6 and in-miR-557 into pancreatic cancer cells, the tumor suppressive functions caused by circEIF6 silencing were largely counteracted by the interference of miR-557. These results demonstrated that circEIF6 exerted its functions partly based on its regulatory relationship with miR-557.

As predicted by TargetScan and verified by dual-luciferase reporter assay, *SLC7A11* was confirmed as one of the targets of miR-557. *SLC7A11* was reported to regulate the extracellular cysteine transportation.^{15,17} Accumulating evidence has been uncovered that aberrant expression of *SLC7A11* is related to the preneoplastic lesions and the development of many malignancies.^{34,35} A high level of *SLC7A11* was observed in many malignancies, and high abundance of *SLC7A11* was associated with dismal prognosis and chemoresistance of cancer cells.^{36,37} For instance, Wu et al claimed that *SLC7A11* expression was enhanced in oral squamous cell carcinoma tissues and cell lines, and the forced expression of miR-375 suppressed cell proliferation and invasion of oral squamous cell carcinoma cells through suppressing *SLC7A11*.³⁸ Zhu et al demonstrated that miR-139-5p hampered the proliferation and motility of pancreatic cancer cells through suppressing *SLC7A11*, thus inactivating PI3K/AKT signaling.¹⁹ *SLC7A11* was observed to be up-regulated in pancreatic tumor tissues and cell lines. Furthermore, circEIF6 silencing reduced the expression of *SLC7A11* through elevating miR-557 expression in pancreatic cancer cells. MiR-557 overexpression restrained the proliferation, migration and invasion and

facilitated the apoptosis of pancreatic cancer cells, while *SLC7A11* overexpression largely regained these malignant behaviors in miR-557-overexpressed pancreatic cancer cells. CircEIF6 silencing also prominently hampered the growth of pancreatic cancer xenograft tumors in vivo.

In conclusion, circEIF6 accelerated the proliferation, migration, invasion and restrained the apoptosis of pancreatic cancer cells through targeting miR-557/*SLC7A11*/PI3K/AKT signal axis. CircEIF6/miR-557/*SLC7A11*/PI3K/AKT signaling might provide novel therapeutic targets for pancreatic cancer therapy.

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Disclosure

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