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Circular RNA ciRS-7 promotes the proliferation and metastasis of pancreatic cancer by regulating miR-7-mediated EGFR/STAT3 signaling pathway

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ABSTRACT

Background: Pancreatic ductal adenocarcinoma (PDAC) is the most deadly type of tumor, and its pathogenesis remains unknown. Circular RNAs (circRNAs) may be functional and bind to microRNAs and consequently, influence the activity of targeted mRNAs. Recent researches indicate that one circRNA, ciRS-7, acts as a sponge of miR-7 and thus, inhibits its activity. It is well known that miR-7 is a cancer suppressor in many cancers. However, the relationship between ciRS-7 and miR-7, and the role of ciRS-7 in PDAC, remains to be elucidated.

Methods: miR-7 and ciRS-7 expression in 41 pairs of PDAC tumors and their paracancerous tissues were detected by quantitative reverse transcription polymerase chain reaction (qRT-PCR). The relationships between their expression levels and clinicopathological features in PDAC tissues were assessed. The relationship between miR-7 and ciRS-7 was also assessed by Spearman's correlation. We also used cell lines to evaluate the role of ciRS-7 in cell line behavior. The ciRS-7 interfere RNA (siRNA) and its empty vector were transfected into PDAC cells. PDAC cells proliferation and invasion abilities were detected by MTT assay and invasion analysis. The expression of proteins was assessed by Western blotting.

Results: ciRS-7 expression was significantly higher in PDAC tissues than paracancerous tissues ($P=0.002$). However, miR-7 expression showed the opposite trend ($P=0.048$). Moreover, ciRS-7 expression was inversely correlated with miR-7 in PDAC ($r_s = -0.353$, $P=0.023$). ciRS-7 expression was also significantly elevated in venous invasion (3.72 ± 2.93 vs. 2.14 ± 1.26; $P=0.028$) and lymph node metastasis (4.19 ± 2.75 vs. 2.32 ± 1.90; $P=0.016$) in PDAC patients. Furthermore, ciRS-7 knockdown suppressed cell proliferation and invasion of PDAC cells ($P < 0.05$), and the downregulation of ciRS-7 resulted in miR-7 overexpression and subsequent inhibition of epidermal growth factor receptor (EGFR) and signal transducer and activator of transcription 3 (STAT3).

Conclusions: Circular RNA ciRS-7 plays an oncogene role in PDAC, partly by targeting miR-7 and regulating the EGFR/STAT3 signaling pathway.

Introduction

Pancreatic ductal adenocarcinoma (PDAC) is a highly malignant tumor with high mortality. PDAC is the fourth main cause of cancer related death in the United States, with a 5-year overall survival rate of less than 5% [1]. Most PDAC patients have poor outcomes because of difficulties in early diagnosis and lack of effective therapies [2–5]. The specific pathogenesis of PDAC is still unclear.

Circular RNAs (circRNAs) are broad and diverse endogenous RNAs that regulate target gene expression at the transcriptional or post-transcriptional level [6,7] via interacting with other molecules or microRNAs (miRNAs). Previous researches have shown that circRNAs play a crucial role in many biological processes such as cell proliferation and invasion [8,9]. Recent studies have demonstrated that circRNAs act as inhibitors and sponges of miRNAs in many cancers [10,11]. In particular, ciRS-7 was demonstrated to act

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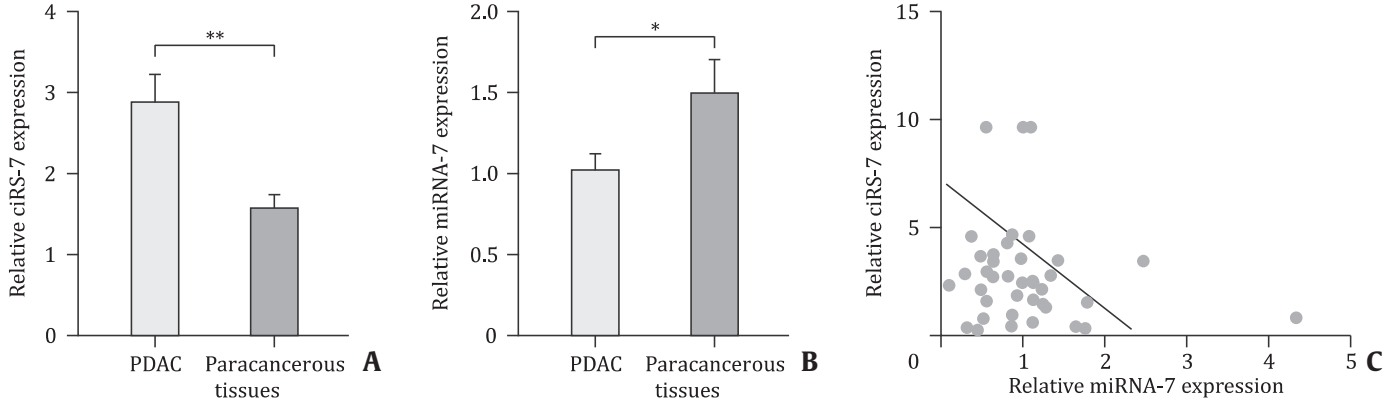

Fig. 1. ciRS-7 expression was upregulated in PDAC tissues and was negatively correlated with miR-7 expression. **A:** The expression of ciRS-7 in PDAC tissues was higher than that in paracancerous tissues ($P = 0.002$); **B:** The expression of miR-7 in PDAC tissues was lower than that in paracancerous tissues ($P = 0.048$); **C:** ciRS-7 expression was inversely correlated with miR-7 expression in PDAC ($P = 0.023$).*: $P < 0.05$; **: $P < 0.01$.

as a strong miR-7 sponge in hepatocellular cancer and colorectal tumors, suggesting a novel mechanism of miRNA function regulation [12,13]. miR-7 is an endogenous noncoding RNA molecule. Multiple studies have revealed that miR-7 is low expressed in many types of tumors, and is negatively correlated with tumor growth and invasion [14–16]. miRNAs may also participate in various biological processes by regulating the proliferation and migration of tumor cells [17,18]. In gynecologic oncology, miR-7 targets epidermal growth factor receptor (EGFR), inactivates protein kinase B (AKT)/extracellular signal-regulated kinase (ERK1/2), reverses epithelial-mesenchymal transition (EMT) and inhibits tumor metastasis [19]. This study aimed to determine whether ciRS-7 affects cell proliferation and migration in PDAC, and to investigate the potential underlying mechanisms.

Methods

Patients and PDAC samples

This study was approved by the Biomedical Ethics Committee of Anhui Provincial Hospital, and the consent form was obtained from all patients. A total of 41 PDAC tumors and their paracancerous tissues were collected from patients who underwent radical surgery between January 2017 and October 2017. The fresh tissue specimens were collected immediately after pancreatic resection and stored at −80 °C, and paracancerous tissues were defined as pancreatic tissues that were more than 2 cm distant from the tumor margin. The clinical and pathological parameters of the patients were obtained from the Electronic Information System of Anhui Provincial Hospital.

Quantitative reverse transcription polymerase chain reaction (qRT-PCR)

The total RNA was extracted from PDAC and paracancerous tissues using TRIzol reagent (Life Technologies, Carlsbad, USA) following the instruction. The expression levels of miR-7 and ciRS-7 were quantified by qRT-PCR. The combination of primer 5.0 software (Premier Biosoft, Palo Alto, USA) and Sangon Biotech (Shanghai, China) was used to design all primers (Table 1). We used the $2^{-\Delta\Delta Ct}$ method to calculate the relative expression levels of each gene.

Cell culture and transfection

BXPC-3, PANC-1 and HPC-Y5 cell lines were from the cell resources center of Shanghai Academy of Sciences, Chinese Academy of Sciences. All the cells were cultured in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal bovine serum (FBS) at 37 °C in a humidified atmosphere containing 5% CO_2. The small interfering RNA (siRNA, siciRS-7-1 and siciRS-7-2) were from Sangon Biotech. The ciRS-7 was knocked down by siRNA and its empty vector were used as negative control and transfected into BXPC-3 cells using lipofectamine 2000 (Invitrogen, Carlsbad, USA) according to the instruction.

Table 1
Primer sequences for qRT-PCR.

Primers	Forward (5′–3′)	Reverse (5′–3′)
ciRS-7	GTCTTCCAACAACTACCAGT	GTGCCATCGGAAACCCTGGA
miR-7	ATGGTTCGTGGGTGGAAGAC	GTCGTATCCAGTGCAGGGTCCG
GAPDH	CATGTACGTTGCTATCCAGG	CTCCTTAATGTCACGCACGA
U6	CTCGCTTCGGCAGCACA	AACGCTTCACGAATTTGCGT
EGFR	CCTCCCATGCGCCGCCCCAC	GGGAATGGAAAGAAAAGGAG
STAT3	GCGCTGCCCCATACCTGAAG	GTTTGACGGGAGGACGACTC

MTT assay and invasion analysis

After transfection or treatment, the cells were seeded on 96-well plates with 1.0×10^4 cells per well and cultured. At 24 h, 48 h and 72 h, an MTT solution with a final concentration of 0.5 mg/mL was added to each well. After incubation for an additional 4 h, the supernatant was removed, and 150 μL dimethyl sulfoxide (DMSO; Sigma-Aldrich, St. Louis, MO, USA) was added to each well. Finally, the optical density at 492 nm was measured by a microplate reader (Life Technologies, Carlsbad, CA, USA). Cells treated with cyclophosphamide were cultured for 48 h, MTT solution was then added in triplicate. The survival ratio of the cells was calculated. Cell invasion was measured using Transwell Matrigel (BD Biosciences, San Jose, USA). Cells at 1.0×10^4 concentration were seeded on the Transwell inserts. A medium containing 10% FBS was added under the membrane. After incubation for 24 h, cotton swabs were used to remove the uninfected cells on the upper surface of the membrane. The invaded cells were immobilized with 95% ethanol, stained with hematoxylin, counted and photographed under light microscope.

Western blotting

Total protein was extracted from pancreatic cancer cells using RIPA buffer (EMD Millipore, Billerica, USA). The proteins were isolated using 12% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) (Sangon Biotech) and transferred to polyvinylidene fluoride (PVDF) membranes (EMD Millipore). The

PVDF membranes were blocked with 5% milk in tris-buffered saline containing 0.1% Tween 20 (TBST) at 4 °C overnight. After washing with TBST, the membranes were nurtured with primary antibodies against EGFR, signal transducer and activator of transcription 3 (STAT3), and GAPDH (Abcam, Cambridge, UK). The protein bands were detected with X-ray film using chemiluminescent reagents.

Statistical analysis

The measurement data were presented as means ± standard deviation (SD) and a P value <0.05 was considered statistically significant. Differences between groups were estimated using Student's t-test and analysis of variance (ANOVA). Spearman's correlation test was applied to assess the correlation between miR-7 and ciRS-7. All statistical analyses were carried out by SPSS software (V17.0; SPSS, Inc., Chicago, IL, USA).

Results

ciRS-7 expression was upregulated in PDAC and negatively correlated with miR-7 expression

ciRS-7 was detected in all 41 PDAC and their paracancerous tissues using qRT-PCR. Compared with paracancerous tissues, ciRS-7 expression was higher in PDAC ($P=0.002$; Fig. 1A). However, miR-7 expression was lower in PDAC than that in the paracancerous tissues ($P=0.048$, Fig. 1B). The abnormal expression of ciRS-7 has a close relation with invasion and metastasis of pancreatic cancer.

Because ciRS-7 has been demonstrated to act as a powerful miR-7 sponge in hepatocellular cancer and colorectal tumors, we conducted further analysis and found that miR-7 expression was negatively correlated with ciRS-7 in PDAC tissues ($r_s = -0.353$, $P=0.023$; Fig. 1C).

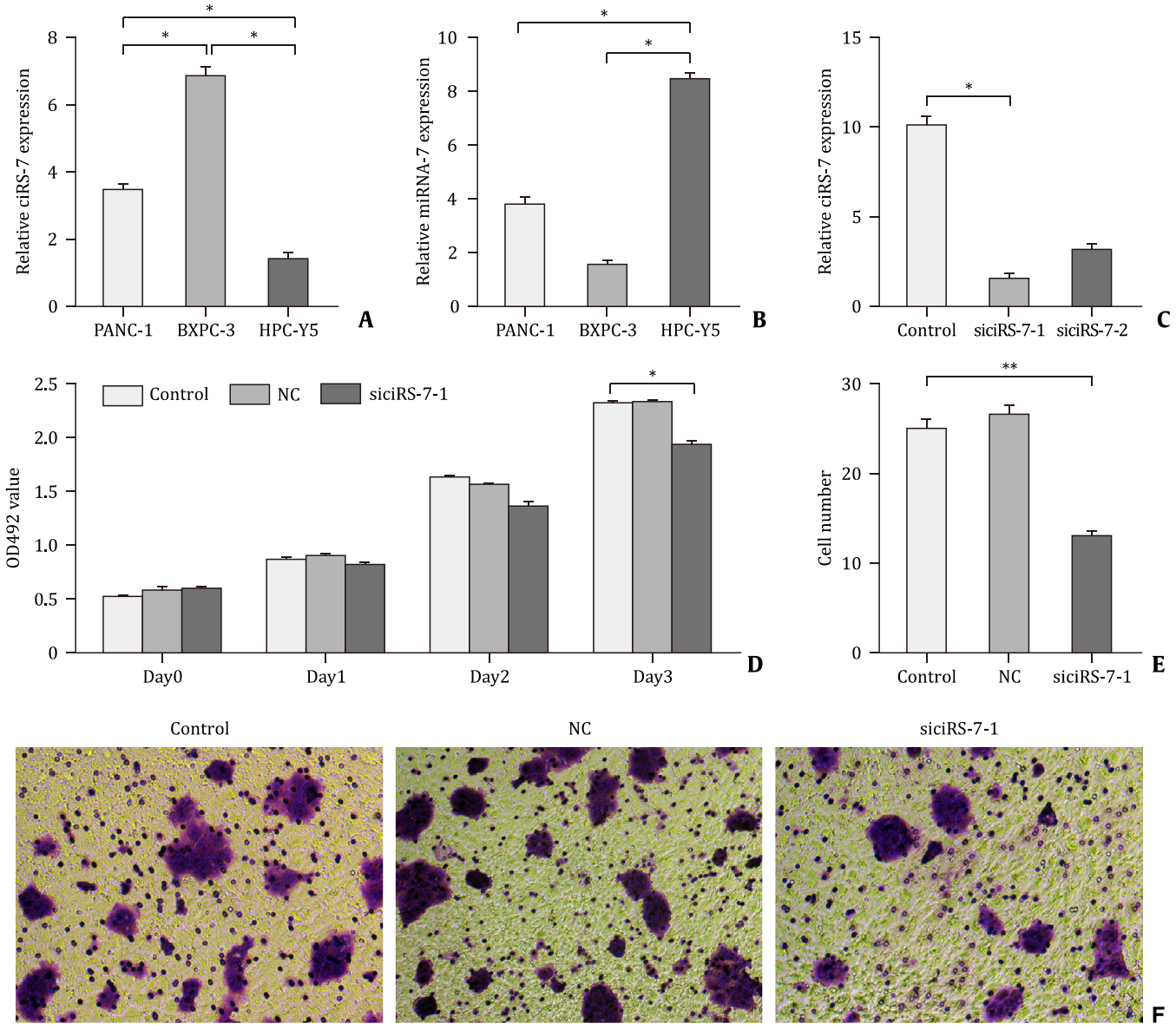

Fig. 2. ciRS-7 knockdown suppressed PDAC proliferation and invasion. **A:** The expression of ciRS-7 in PDAC cells was higher than that in human pancreatic cells; **B:** miR-7 expression showed the opposite trend; **C:** ciRS-7 knockdown suppressed ciRS-7 expression in BXPC-3 cells; **D:** siciRS-7-1 inhibited the proliferation of BXPC-3 cells; **E:** siciRS-7-1 inhibited BXPC-3 invasion; **F:** representative images of siciRS-7-1 inhibiting the invasion of BXPC-3 cells (original magnification × 400). Control: blank control, non-transfected cells group; NC: negative control, empty vector group.*: $P < 0.05$; **: $P < 0.01$.

The relationship between ciRS-7 levels and clinicopathological parameters in PDAC

The relationship between ciRS-7 levels and clinicopathological parameters in PDAC tissues are listed in Table 2. Patients with lymph node metastasis had significantly higher ciRS-7 levels in tumor tissues compared with those without lymph node metastasis (4.19 ± 2.75 vs. 2.32 ± 1.90; $P = 0.016$). Patients with tumor venous invasion had also significantly increased ciRS-7 levels in tumor tissues (3.72 ± 2.93 vs. 2.14 ± 1.26; $P = 0.028$). The study showed that the expression of ciRS-7 in PDAC was related to the grade of tumor malignancy.

ciRS-7 knockdown suppressed PDAC proliferation and invasion

ciRS-7 was upregulated in PDAC cells (PANC-1 and BXPC-3) compared with that in human pancreatic cells (HPC-Y5). However, miR-7 expression showed the opposite trend (Fig. 2A and B). Because the expression level of ciRS-7 was the highest in BXPC-3 cells, we used BXPC-3 cells in subsequent experiments. We also confirmed that ciRS-7 knockdown (siciRS-7) suppressed ciRS-7 expression, and siciRS-7-1 had higher inhibition efficiency (Fig. 2C). Further investigation showed that siciRS-7-1 inhibited the proliferation of pancreatic cancer cell line BXPC-3 (Fig. 2D). Additionally, siciRS-7-1 inhibited BXPC-3 invasion (Fig. 2E and F).

ciRS-7 knockdown influenced the expression of miR-7 and its target gene, EGFR and STAT3

Our results indicate that siciRS-7-1 promoted miR-7 expression in PDAC (Fig. 3A). We further explored the mechanism underlying the effects of ciRS-7 on the biological characteristics of pancreatic cancer through miR-7. Using the miRNA target analysis tool TargetScan, we found that EGFR was a possible target of miR-7. To assess and confirm whether ciRS-7 promotes its oncogenic potential by suppressing miR-7 activity and regulating the EGFR/STAT3

Table 2
Relationship between ciRS-7 expression in PDAC tissues and clinical characteristics.

Characteristics	*n*	ciRS-7 relative expression	
		Values	*P* value
Sex			0.248
Male	26	2.55 ± 2.03	
Female	15	3.43 ± 2.72	
Age (yr)			0.827
<60	8	3.03 ± 3.04	
≥60	33	2.83 ± 2.15	
Tumor location			0.755
Head	17	2.73 ± 1.38	
Body and tail	24	2.97 ± 2.81	
Tumor diameter (cm)			0.306
<5	24	2.56 ± 2.08	
≥5	17	3.31 ± 2.60	
CA19-9 (ng/μL)			0.824
<200	19	2.96 ± 2.03	
≥200	22	2.80 ± 2.57	
Diabetes			0.911
Yes	20	2.91 ± 2.04	
No	21	2.83 ± 2.59	
Lymph node metastasis			**0.016**
Positive	12	4.19 ± 2.75	
Negative	29	2.32 ± 1.90	
Venous invasion			**0.028**
Positive	19	3.72 ± 2.93	
Negative	22	2.14 ± 1.26	

PDAC: pancreatic ductal adenocarcinoma.

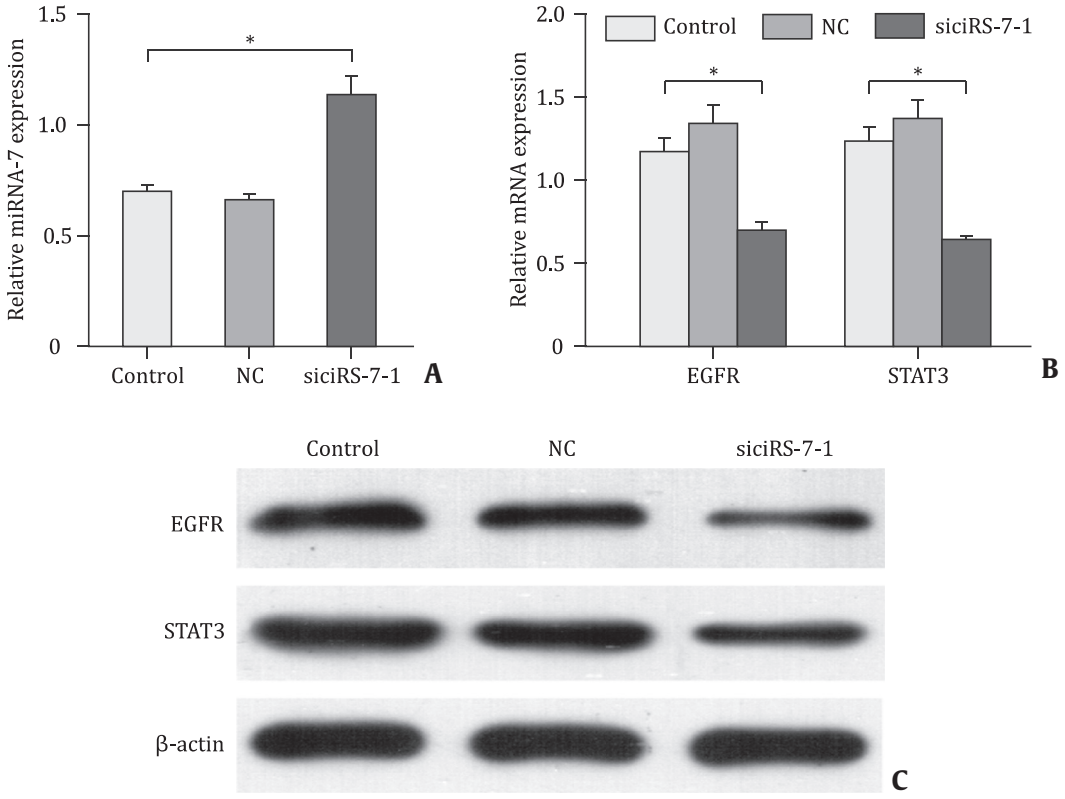

Fig. 3. ciRS-7 knockdown promoted miR-7 expression and inhibited the expression of its target genes, EGFR and STAT3 in BXPC-3 cells. **A:** ciRS-7 knockdown promoted miR-7 expression; **B:** qRT-PCR detection showed that siciRS-7-1 suppressed the expression of EGFR and STAT3; **C:** Western blotting analysis showed that siciRS-7-1 suppressed the expression of EGFR and STAT3. Control: blank control, non-transfected cells group; NC: negative control, empty vector group. *: $P < 0.05$.

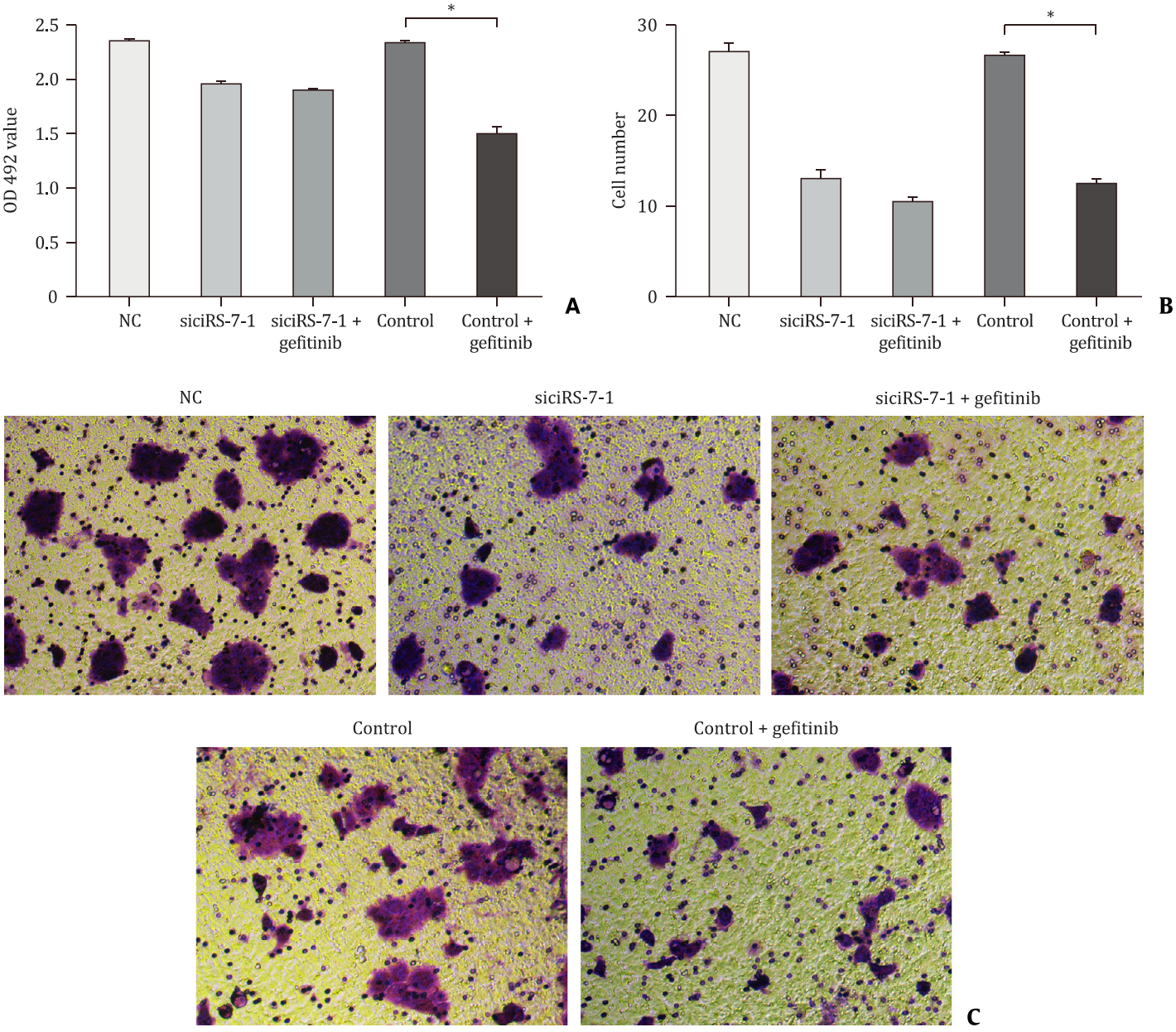

Fig. 4. The selective EGFR inhibitor gefitinib had little effect on the invasion and proliferation of siciRS-7-1-transfected BXPC-3 cells. **A:** Gefitinib treatment had little effect on the proliferation of siciRS-7-1-transfected BXPC-3 cells, but significantly decreased the proliferation of control cells; **B:** Gefitinib treatment had little effect on the invasion of siciRS-7-1-transfected BXPC-3 cells, but significantly decreased the invasion of control cells; **C:** Representative images of BXPC-3 cell invasion (original magnification × 400). Control: blank control, non-transfected cells group; NC: negative control, empty vector group. *: $P < 0.05$.

signaling pathway, we detected the expression of EGFR and STAT3 in the siciRS-7-1 group and control group cells. As expected, we observed that siciRS-7-1 also suppressed the expression of EGFR and STAT3 (Fig. 3B and C).

The selective EGFR inhibitor gefitinib had little influence on the invasion and proliferation of siciRS-7-1 group cells

The siciRS-7-1 group and control group cells were treated with the same concentration of EGFR inhibitor gefitinib (10 μmol/L; Abcam). The results showed that gefitinib had little influence on the proliferation and invasion of siciRS-7-1 transfected BXPC-3 cells. However, gefitinib treatment significantly reduced the proliferation and invasion of the control group ($P < 0.05$; Fig. 4).

Discussion

circRNA is a special type of non-coding RNA and is also a potential cancer research target. To date, ciRS-7 is one of the few known circRNAs that has been proposed to inhibit tumor suppressor miR-7 [20]. In the current research, we found that ciRS-7 is frequently upregulated in PDAC, and also displayed that the degree of expression of ciRS-7 was positively related to two clinicopathological characteristics: lymph node metastasis and venous invasion. In addition, we found that the expression of ciRS-7 was negatively correlated with miR-7 in PDAC. From a biological perspective, we believe that the overexpression of ciRS-7 suppresses the expression of miR-7, and ciRS-7 has a potential carcinogenic effect in PDAC.

To better understand the role of ciRS-7 in PDAC, we knocked down ciRS-7 in pancreatic cancer cell line and found that ciRS-7

knockdown inhibited the proliferation and invasion of BXPC-3 cells, and that ciRS-7 knockdown promoted miR-7 expression in PDAC. Previous studies have suggested that miR-7 may play an important regulatory role in inhibiting the progression of pancreatic cancer by damaging the autophagic glucose pool [21]. Accordingly, in our study, the cytological study supported the histological results; ciRS-7 knockdown inhibited the proliferation and invasion of PDAC cells partly by targeting miR-7.

Dysregulation of signaling pathways plays a pivotal role in the proliferation and invasion of PDAC [22–24]. A recent study revealed that ciRS-7 overexpression blocked the tumor suppressive effect of miR-7 on MGC-803 and HGC-27 cells, and resulted in more aggressive carcinogenic phenotypes via antagonizing miR-7-PTEN/PI3K/AKT pathway [25]. Another study discovered that the ciRS-7/miR-7/NF-κB axis exerted pronounced effects on the proliferation, migration, invasion, and apoptosis of non-small cell lung cancer (NSCLC) cells [26]. To investigate whether ciRS-7 exerts its oncogenic effects by suppressing miR-7 activity and regulating signaling pathways, Suto and colleagues used the miR target analysis tool TargetScan and found that EGFR was a potential target of miR-7 [27]. EGFR is a transmembrane glycoprotein with tyrosine kinase activity, which has a cytoplasmic domain. Specific ligand binding sites, including EGF, activate downstream signaling pathways and are involved in cell proliferation and survival [28,29]. The EGFR pathway plays an important role in many tumor progression, such as in breast, colon, and gastric cancer [30–32]. Previous researches have demonstrated that EGFR upregulation occurs in 30%–50% of human pancreatic tumors, and is correlated with rapidly progressive disease, resistance to chemotherapy, and poor prognosis [33,34]. Studies have also found that miR-7 attenuates the activation of EGFR/IGF-1R and EGFR/PI3K/AKT signaling and EGFR-associated signaling pathways [35,36], thereby inhibiting tumor cell proliferation and inhibiting tumor invasion. In this study, we observed that ciRS-7 knockdown suppressed the expression of EGFR and STAT3 (Fig. 3B and C). Our results showed that the EGFR/STAT3 signaling pathway plays an essential role in the proliferation and invasion of PDAC. To further verify whether ciRS-7 influences the biological characteristics of pancreatic cancer through EGFR/STAT3 signaling pathway, we transfected cells with siciRS-7-1 and treated these cells and control cells with the same concentration of the EGFR inhibitor gefitinib. The results suggest that gefitinib had little influence on the proliferation and invasion of siciRS-7-1 transfected cells; however, there was a significant reduction in both processes in the control group. From another point of view, these results showed that ciRS-7 affected the proliferation and invasion of PDAC cells through the EGFR/STAT3 signaling pathway.

In conclusion, this study showed that ciRS-7 exerts its oncogenic effects by inhibiting miR-7 activity and consequently activating EGFR/STAT3 signaling pathway in PDAC. Cytological studies demonstrated that ciRS-7 knockdown affected PDAC cell proliferation and invasion. Our results suggest that ciRS-7 plays a carcinogenic role in PDAC and that the EGFR/STAT3 signaling pathway may have an extremely important impact on the proliferation and invasion of PDAC.

Contributors

LCH and HQ proposed the study. LL, LFB and XQS performed the research and wrote the first draft. HM, XK and SMJ collected and analyzed the data. All authors contributed to the design and interpretation of the study and to further drafts. HQ is the guarantor.

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Ethical approval

This study was approved by the Biomedical Ethics Committee of Anhui Provincial Hospital (2017-0034).

Competing interest

No benefits in any form have been received or will be received from a commercial party related directly or indirectly to the subject of this article.

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