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MicroRNA-374a promotes pancreatic cancer cell proliferation and epithelial to mesenchymal transition by targeting SRCIN1

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ABSTRACT

MicroRNAs (miRNAs) play a vital role in the progress of cancer. Whereas the expression and function of miR-374a in pancreatic cancer remain largely unknown. In this study, pancreatic cancer samples and its adjacent normal tissues were obtained from 30 clinical patients with pancreatic cancer. Quantitative real-time PCR (qRT-PCR) was used to measure miR-374a and SRC Kinase Signaling Inhibitor 1 (SRCIN1) expression. Western blotting assay was performed to measure the levels of SRCIN1, E-cadherin, N-cadherin, Vimentin, Zonula occludens-1 (ZO-1) and β-catenin in PANC-1 cells. Luciferase reporter assay was conducted to confirm the direct targeting of SRCIN1 by miR-374a. Cell proliferation and migration assays were utilized to analyze the role of miR-374a in PANC-1 cells. We found that miR-374a expression was upregulated in pancreatic cancer tissues and cell lines. Over-expression of miR-374a promoted cell proliferation, migration and epithelial-mesenchymal transition (EMT) in pancreatic cancer. While, SRCIN1 expression was downregulated in pancreatic cancer tissues and cells. SRCIN1 was found to be a potential targets of miR-374a by dual-luciferase reporter assay. And SRCIN1 was down-regulated after miR-374a transfection. More than that, over-expression of SRCIN1 inhibited cell proliferation, migration and EMT in pancreatic cancer cell. Therefore, this study revealed that miR-374a promoted cell proliferation, migration and EMT via targeting SRCIN1 in pancreatic cancer.

1. Introduction

According to reports, there are approximately 227,000 cases of pancreatic cancer deaths worldwide each year [1]. And pancreatic cancer will become the second lethality cancer by 2030 [2]. About half of patients with pancreatic cancer patients have metastasis, which is closely associated with the high mortality [3,4]. Surgical resection was thought to be the only potentially permanent therapy for pancreatic cancer. However, the 5-year survival rate is only approximately 20% in patients with surgery [5]. The overall 5-year survival rate is only 6% after diagnosis [6]. Because of some pancreatic cancer patients can only be diagnosed after tumor metastasized [7]. It is urgent to understand the molecular mechanisms of pancreatic cancer progression, which will improve the treatment of pancreatic cancer.

MicroRNAs (MiRNAs) are a group of endogenous small noncoding RNAs, which contain about 20–24 nucleotides [8,9]. It was proved that miRNAs bind to target genes by targeting the 3′-untranslated regions (3′UTR) and regulate the translation and degradation of target mRNAs [10,11]. A large number of miRNAs have been proven to be essential in various malignant tumors, including pancreatic cancer [12–14]. Recently, miR-374a has been shown to be up-regulated in multiple types of cancer, including non-small cell lung carcinoma (NSCLC) [15], breast cancer [16], osteosarcoma [17] and colon cancer [18]. MiR-374a promoted metastasis and epithelial-mesenchymal transition (EMT) by suppressing Wnt inhibitory factor 1 (WIF1), PTEN or Wnt5A expression in breast cancer [19]. MiR-374a directly targets Wnt5a and acts as an oncogene to regulate proliferation, apoptosis, migration, invasion and EMT of NSCLC [20]. However, the expression and role mechanisms of miR-374a in pancreatic cancer are largely unknown.

SRC Kinase Signaling Inhibitor 1 (SRCIN1) has been shown to be down-regulated in cancer [21]. miR-374a promoted cell proliferation, invasion and migration by targeting SRCIN1 in gastric cancer [22]. miRNA-32 promoted proliferation and EMT in human liver cancer cells by downregulation of SRCIN1 [23]. MiR-873 could target SRCIN1 and induced proliferation and migration in lung adenocarcinoma cells [24].Over-expression of SRCIN1 inhibited the growth and metastasis of colorectal cancer [25]. However, the expression and role mechanisms of SRCIN1 in pancreatic cancer are largely unknown.

In the present study, we demonstrated that over-expression of miR-374a promoted the proliferation, migration and EMT in pancreatic

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cancer cells. Importantly, we further found that miR-374a negatively regulated the target gene expression of SRCIN1, and over-expression of SCIN1 inhibited cell proliferation, migration and EMT in pancreatic cancer cell. Thus, miR-374a promoted cell proliferation, migration and EMT via targeting SRCIN1 in pancreatic cancer cell.

2. Materials and methods

2.1. Patients and tissue samples

A total of 30 pairs of pancreatic cancer tissues and corresponding matched adjacent non-cancerous tissues were collected from patients for pancreatic cancer surgery. Both computed tomography and MR are used to detect pancreatic cancer [26]. Pancreatic adenocarcinoma staging is based on tumor size, location within the pancreas, involvement of surrounding vessels, and presence of metastatic disease [27]. Fresh tissue were taken within 10 min of tumor excision, immediately immersed in RNAlater™ Stabilization Solution (Thermo Fisher Scientific, Waltham, MA, USA) and stored at −80 °C until use.Our research has been approved by the Medical Ethics Committee. And the informed consent and patient anonymity were also available from the patients.

2.2. Cell culture and transfection

The HPAC, PANC-1 human pancreatic cancer cell lines and HPDE6-C7 normal human osteoblast cell line were obtained from the Type Culture Collection of the Chinese Academy of Sciences (Shanghai, China). The cells were cultured in Dulbecco modified Eagle medium (DMEM) added with 10% fetal bovine serum (FBS) at 37 °C under 5% CO_2 humidified incubator. The cells were transfected with miR-374a mimics, miR-374a-inhibitors, miR-374a negative control (miR-NC), SRCIN1 overexpression plasmid and negative control using lipofectamine 2000 transfection reagent (Invitrogen, Carlsbad, CA, USA) in six-well plates following the manufacture's protocol.

2.3. Quantitative Real-time polymerase chain reaction (qRT-PCR)

The tissues and cells samples were lysed in Trizol (Invitrogen, Carlsbad, CA, USA) for total RNA extraction. Real-time PCR was performed using a universal reverse primer as well as the specific forward primer for miR-374a. U6 and GAPDH were used as the internal control of the miR-374a and SRCIN1. The primer sequences are as follows: miR-374a forward: GCCGTTTATAATACAACCTGA-3; SRCIN1 forward: 5′-GAGGCTCGCAACGTCTTCTAC-3′ and reverse: 5′-GCGATGCGTACA CCATCTCTC-3′; U6 forward: 5′-CTCGCTTCGGCAGCACA-3′ and reverse: 5′-AACGCTTCACGAATTTGCGT-3′; GAPDH forward: 5′- GGAGC GAGATCCCTCCAAAAT-3′ and reverse: 5′- GGCTGTTGTCATACTTCTC ATGG-3′. $2^{-\Delta\Delta Ct}$ method was used in each sample as relative quantification.

2.4. Cell proliferation

Cell Counting Kit-8 (CCK-8, Dojindo Laboratories, Kumamoto, Japan) was applied to evaluate the cell proliferation. Briefly, cells transfected with miR-374a mimics, miR-374a-inhibitors, miR-NC and SRCIN1 were seeded into 96-well plates (5×10^3 cell/well) with 100 µl medium. And CCK-8 reagents were added into each well at 24 h, 48 h and 72 h by incubating at 37 °C. The optical density (OD) was measured at 450 nm on an enzyme-linked immunosorbent assay reader.

2.5. Cell migration

Cell migration assays were performed using 24-well culture plates inserted with 8-mm pore size culture (Transwell; Falcon, BD Biosciences). The lower chamber was filled with 600 µl DMEM containing 10% FBS. Cells (1×10^5 cell/well) were seeded to the upper chamber. After 24 h incubation, the number of the bottom well cells were counted by counting chamber.

2.6. Western blot analysis

The cells and samples were collected and lysed at 48 h after treatment using RIPA buffer. The same amounts of proteins were resolved by 10–15% SDS-PAGE and then transferred to a PVDF membrane (Millipore Corporation, Billerica, MA, USA). The membrane was incubated with specific antibodies (SRCIN1, E-cadherin, N-cadherin, Vimentin, ZO-1 and β-catenin; Cell Signaling Technology Inc.) and peroxidase-conjugated secondary antibodies (KPL, Gaithersburg, MD, USA) at 4 °C overnight. Chemiluminescence (Milipore Corporation) and densitometry analysis (ImageJ software) were applied to measure the protein expression.

2.7. Luciferase reporter assay

In the luciferase reporter assay, the PANC-1 cells were cotransfected with control + SRCIN1 WT, miR-NC + SRCIN1 WT, miR-374a + SRCIN1 WT, pGL3-control + SRCIN1 MUT, pGL3-miR-NC + SRCIN1 MUT, pGL3-miR-374a + SRCIN1 MUT plasmids using Lipofectamine 2000. Reporter activity was detected by a luciferase assay kit according to the manufacturer instructions.

2.8. Statistical analysis

All experiments were conducted independently at least three times. All data analysis was performed using GraphPad Prism software version 5.0 (GraphPad, Software, Inc., La Jolla, CA, USA). The difference among groups was evaluated by a paired or unpaired t-test. Data were shown as the mean ± standard deviation. P value less than 0.05 was considered statistically significant.

3. Results

3.1. MiR-374a was significantly upregulated in pancreatic cancer tissues and cells

To study the expression of miR-374a in the development of pancreatic cancer, 30 samples of human pancreatic cancer tissues (Cancer group) and non-cancerous tissues (Paracarcinoma group) were obtained to evaluate the expression of miR-374a using qRT-PCR analysis. Significant upregulation of miR-374a was observed in pancreatic cancer tissues compared to the adjacent normal non-cancerous tissues (Fig. 1A). In addition, the expression of miR-374a in HPAC, PANC-1 cell lines and normal pancreatic ductal epithelial HPDE6 cell were also analyzed using qRT-PCR. As shown in Fig. 1B, expression of miR-374a in HPAC and PANC-1 cells was significantly increased compared with HPDE6 cells. These results confirmed that miR-374a was significantly upregulated in pancreatic cancer tissues and cells. Furthermore, the results revealed that miR-374a might be an oncogenes in pancreatic cancer.

3.2. MiR-374a promoted proliferation, migration and EMT in pancreatic cancer cells

To explore the potential biological function of miR-374a in the progression of pancreatic cancer, the PANC-1 cells were transfected with miR-374a mimics and analyzed by qRT-PCR. miR-374a expression was significantly increased after miR-374a mimics transfection at 24, 48 and 72 h (Fig. 2A). Then, cell proliferation was detected by Cell Count Kit-8 (CCK-8). Compared with the negative control, cell proliferation was significantly increased after transfected with miR-374a mimics (Fig. 2B). Thus, overexpression of miR-374a significantly promoted pancreatic cancer PANC-1 cell proliferation.

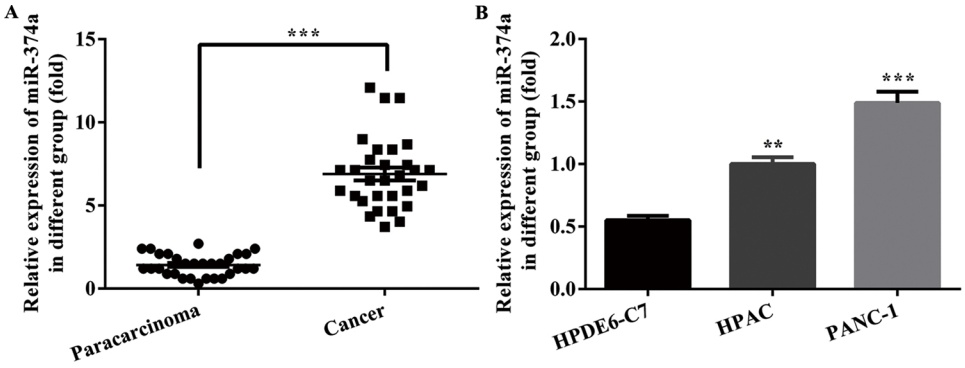

Fig. 1. miR-374a expression was up-regulated in human pancreatic cancer tissues and cell lines. (A) The expression levels of miR-374a were determined in human pancreatic cancer tissues and normal by qRT-PCR assay. $^{***}P < 0.001$ vs. Paracarcinoma (B) The expression levels of miR-374a were determined in human pancreatic cancer cell lines (HPAC and PANC-1 cell lines and normal pancreatic ductal epithelial HPDE6) by qRT-PCR assay. $^{**}P < 0.01$, $^{***}P < 0.001$ vs. HPDE6-C7 cell.

Since migration is the crucial stage in tumor metastasis and development. Next, we investigated the effects of miR-374a on migration of PANC-1 cell lines by Transwell assay. The results indicated overexpression of miR-374a dramatically promoted the cell migration compared with the negative controls in the pancreatic cancer PANC-1 cell lines (Fig. 2C-D).

EMT is one of the most common regulate manners for cancer cell migration [28]. In present study, miR-374a overexpression significantly decreased the epithelial marker of E-cadherin and ZO-1 protein expression, and increased the mesenchymal cell marker of Vimentin, N-cadherin and β-catenin protein expression by western blotting assay, indicating miR-374a promoted the EMT of pancreatic cancer cells (Fig. 2E-F). Thus, our results suggested that miR-374a promoted cell proliferation, migration and EMT in pancreatic cancer.

3.3. MiR-374a negatively regulated the target gene expression of SRCIN1

TargetScan was introduced to search target genes of miR-374a to study the mechanisms of miR-374a promotes proliferation, migration and EMT of pancreatic cancer cell. SRCIN1 was found to be a potential targets of miR-374a. 30 pairs of human OS pancreatic cancer tissues and paracarcinoma tissues were analyzed to determine the expression of SRCIN1 using qRT-PCR analysis and Western Blot. Compared with the paracarcinoma tissues, SRCIN1 was significant downregulated in

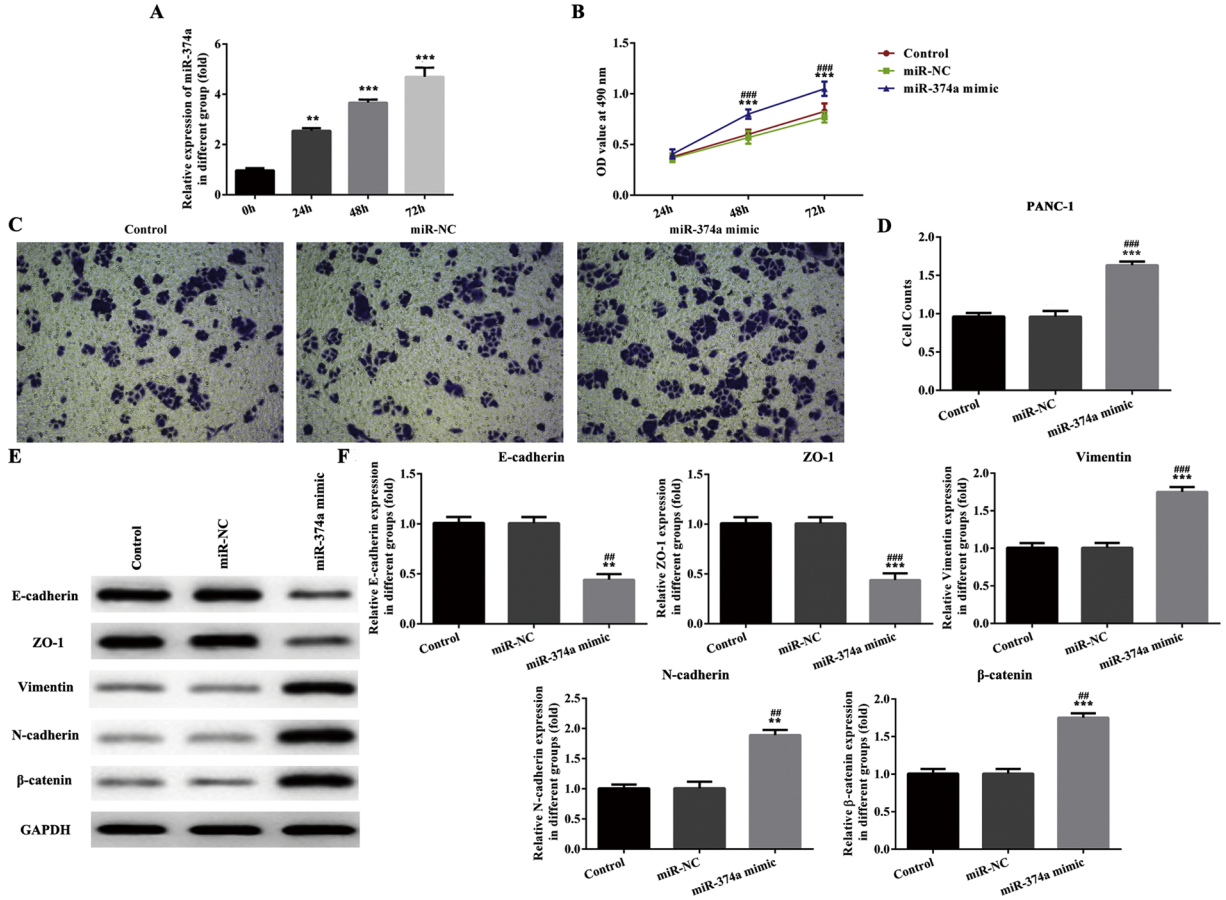

Fig. 2. Overexpression of miR-374a promoted cell proliferation, migration and EMT in PANC-1 cells. (A) PANC-1 cells were transfected with miR-374a mimic for 0 h, 24 h, 48 h and 72 h. The levels of miR-374a were determined by qRT-PCR assay. $^{**}P < 0.01$, $^{***}P < 0.001$ vs. 0 h group. (B) The cells were treated as indicated above, CCK-8 assay results showed that overexpression of miR-374a promoted PANC-1 cell proliferation at 24 h, 48 h and 72 h. $^{***}P < 0.001$ vs. control; $^{###}P < 0.001$ vs. miR-NC. (C) Cell migration was detected using Transwell assay. (D) Migrated cells were counted by counting chamber in each group. $^{***}P < 0.001$ vs. control; $^{###}P < 0.001$ vs. miR-NC. (E–F) E-cadherin, ZO-1, Vimentin, N-cadherin and β-catenin protein expression were determined by western blotting assay and normalized to that GAPDH. $^{**}P < 0.01$, $^{***}P < 0.001$ vs. control; $^{##}P < 0.01$, $^{###}P < 0.001$ vs. miR-NC.

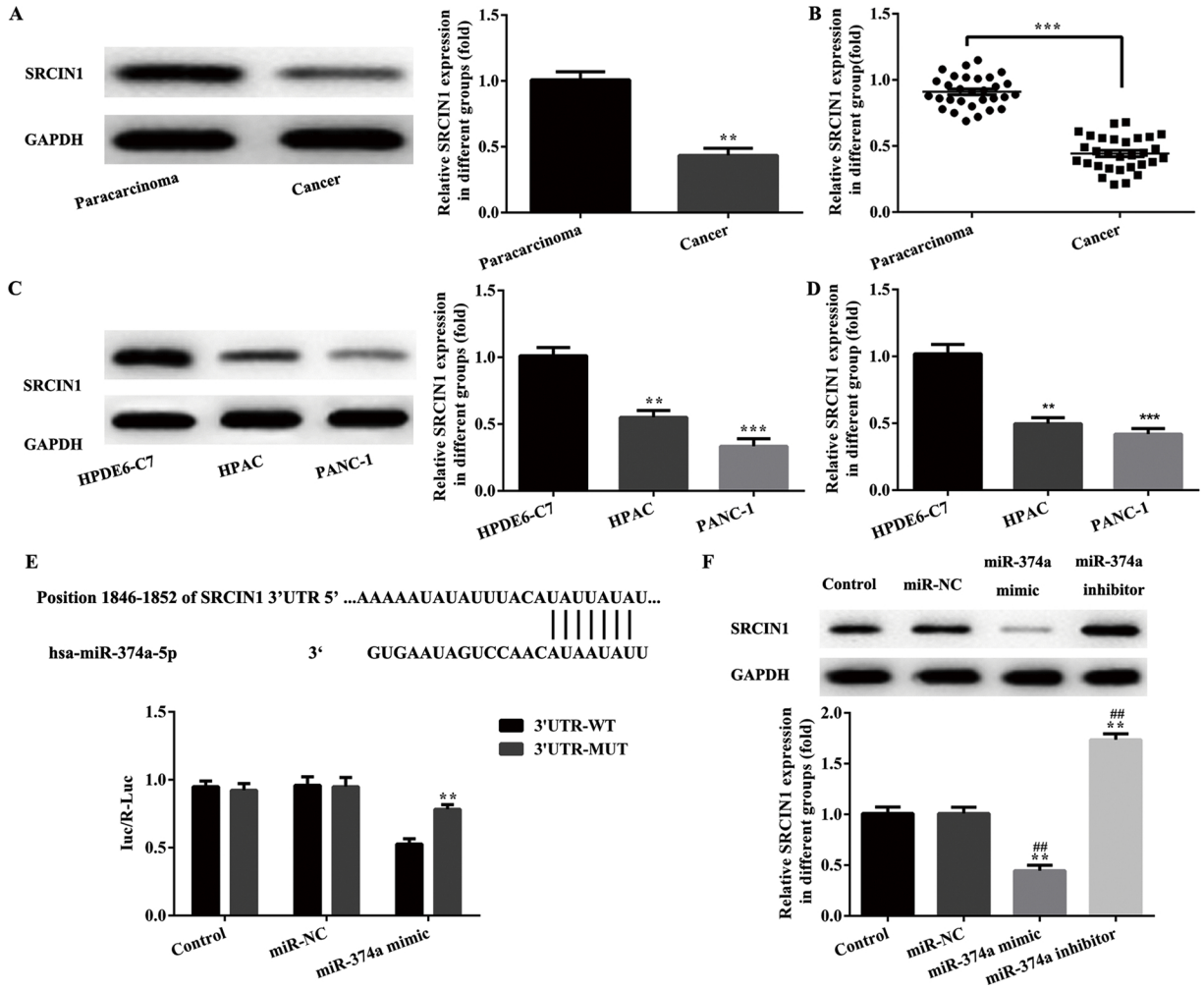

Fig. 3. miR-374a negatively regulated the target gene expression of SRCIN1. (A) The expression of SRCIN1 was detected in human pancreatic cancer tissues and paracarcinoma by Western Blot. $^{**}P < 0.01$ vs. Paracarcinoma group. (B) The expression of SRCIN1 was detected in human pancreatic cancer tissues and paracarcinoma by qRT-PCR. $^{***}P < 0.001$ vs. Paracarcinoma group. (C) The expression levels of SRCIN1 were determined in HPAC, PANC-1 and HPDE6 cell lines by Western Blot. $^{**}P < 0.01$, $^{***}P < 0.001$ vs. HPDE6-C7 cell. (D) The expression levels of SRCIN1 were determined in HPAC, PANC-1 and HPDE6 cell lines by qRT-PCR. $^{**}P < 0.01$, $^{***}P < 0.001$ vs. HPDE6-C7 cell. (E) Luciferase activity were detected in control + SRCIN1 WT, miR-NC + SRCIN1 WT, miR-374a + SRCIN1 WT, control + SRCIN1 MUT, miR-NC + SRCIN1 MUT, miR-374a + SRCIN1 MUT groups by dual-luciferase reporter assays. (F) The expression level of SRCIN1 was measured by Western blot. $^{**}P < 0.01$ vs. control; $^{\#\#}P < 0.01$ vs. miR-NC.

pancreatic cancer tissues and cells (Fig. 3A-B). In addition, SRCIN1 expression was significantly downregulated in HPAC and PANC-1 cells compared with HPDE6 cells (Fig. 3C-D).

Luciferase reporter assays validated that miR-374a could directly target the 3′-untranslated region (UTR) of SRCIN1 compared with NC group (Fig. 3E). Next, Western Blot assay showed that the SRCIN1 protein was decreased in the miR-374a mimics group and was increased by the miR-374a inhibitor (Fig. 3F).In sum, these data revealed that miR-374a directly suppressed SRCIN1 in pancreatic cancer cell.

3.4. MiR-374a promoted cell proliferation, migration and EMT via targeting SRCIN1 in pancreatic cancer cell

To further confirm the hypothesis that miR-374a promote cell proliferation, migration and EMT by targeting SRCIN1 in pancreatic cancer, the transfected NC, miR-374a-mimics and miR-374a + SRCIN1 cells were validated mRNA and protein expression levels of SRCIN1 by Western Blot and qRT-PCR. Compared with miR-374a-mimics group, the mRNA and protein levels of SRCIN1 were rescued in the miR-374a mimic + SRCIN1 (Fig. 4A-B). Compared with miR-374a-mimics group, the cell proliferation significantly inhibited in miR-374a mimic + SRCIN1 group (Fig. 4C). Next, our studies explored the effects of SRCIN1 on PANC-1 cell migration by using Transwell assay. Cell migration significantly inhibited in miR-374a mimic + SRCIN1 group compared with miR-374a mimics group (Fig. 4D-E). We further proved that SRCIN1 overexpression significantly reversed the decreased expression of E-cadherin and ZO-1 and increased expression of Vimentin, N-cadherin and β-catenin protein induced by miR-347a (Fig. 4F-G).Taken these results together, overexpression of SCIN1 inhibited cell proliferation, migration and EMT in pancreatic cancer cell. miR-374a promoted cell proliferation, migration and EMT via binding SRCIN1 in pancreatic cancer cell.

4. Discussion

Previous studies characterized the miRNA expression profiles associated with tumorigenesis, progression and prognosis of pancreatic cancer, and dysregulation of miRNAs play an important role in pancreatic carcinogenesis [29]. MiR-374a was located on chromosome Xq13.2. Here, we validated that expression and role of miR-374a in pancreatic cancer. In previous study, miR-374a was regarded as an oncogene in primary small cell lung cancer [15]. However, miR-374a may also inhibit tumor proliferation in early phase NSCLC [30]. In weakly invasive and metastatic breast cancers, miR-374a was decreased

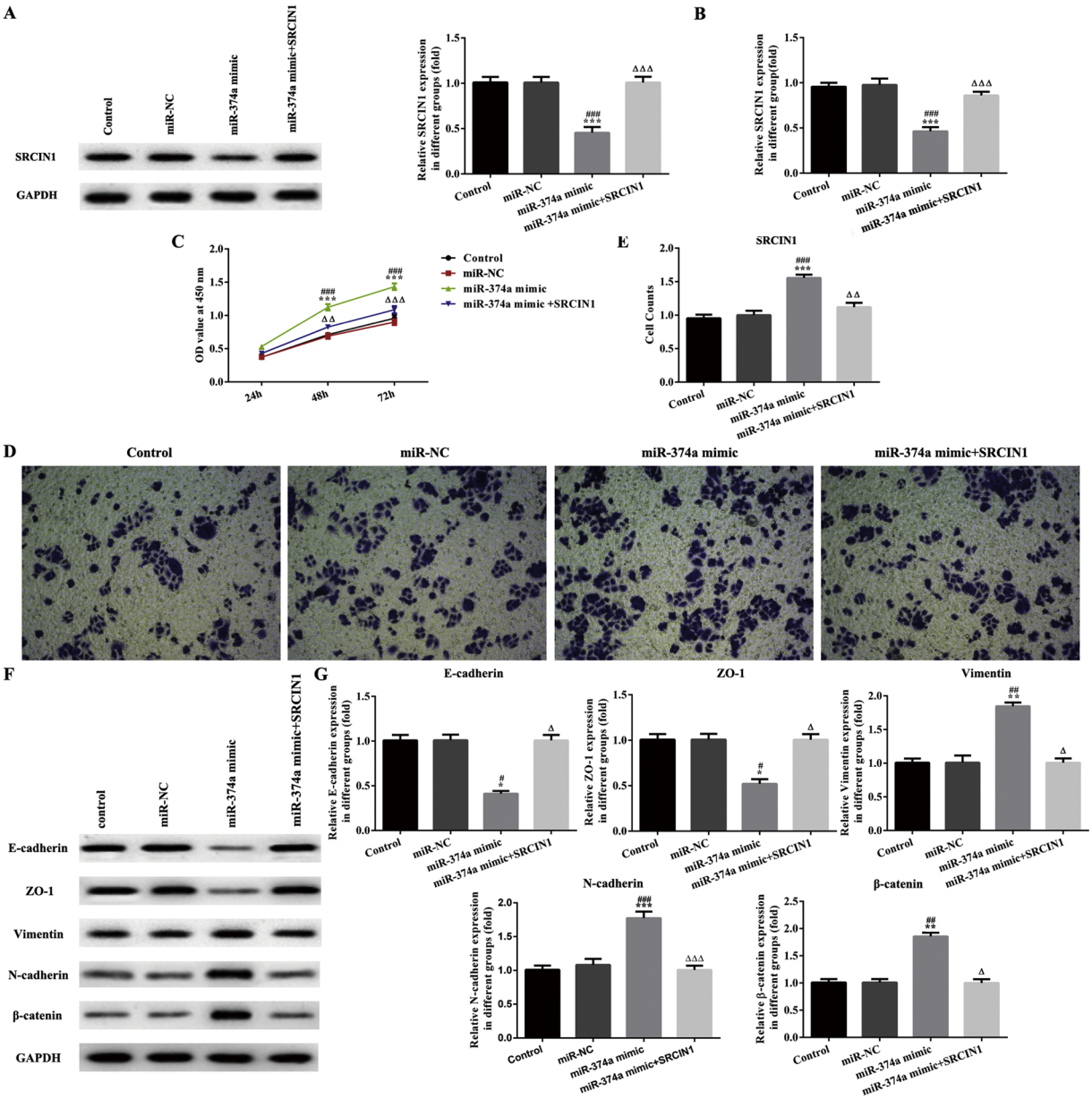

Fig. 4. Overexpression of SRCIN1 significantly inhibited PACN-1 cell proliferation, migration and EMT. (A) The transfected NC, miR-374a-mimic and miR-374a + SRCIN1 cells were validated mRNA and protein expression levels of SRCIN1 by Western blot. $^{***}P < 0.001$ vs. control; $^{###}P < 0.001$ vs. miR-NC; $^{ΔΔΔ}P < 0.001$ vs. miR-374a mimic. (B) The transfected NC, miR-374a-mimic and miR-374a + SRCIN1 cells were validated mRNA and protein expression levels of SRCIN1 by qRT-PCR. $^{***}P < 0.001$ vs. control; $^{###}P < 0.001$ vs. miR-NC; $^{ΔΔΔ}P < 0.001$ vs. miR-374a mimic. (C) CCK-8 assay results showed that overexpression of SRCIN1suppressed PANC-1 cell proliferation. $^{***}P < 0.001$ vs. control; $^{###}P < 0.001$ vs. miR-NC; $^{ΔΔ}P < 0.01$, $^{ΔΔΔ}P < 0.001$ vs. miR-374a mimic. (D) Cell migration were detected using transwell assay. (E) Migrated cells were counted by counting chamber in each group. $^{***}P < 0.001$ vs. control; $^{###}P < 0.001$ vs. miR-NC; $^{ΔΔ}P < 0.01$ vs. miR-374a mimic. (E) E-cadherin, ZO-1, Vimentin, N-cadherin and β-catenin protein expression were determined by western blotting assay. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ vs. control; $^{#}P < 0.05$, $^{##}P < 0.01$, $^{###}P < 0.001$ vs. miR-NC; $^{Δ}P < 0.05$, $^{ΔΔΔ}P < 0.001$ vs. miR-374a mimic.

compared with normal tissues and cells [31]. In order to evaluate miR-374a expression in pancreatic cancer. 30 samples of human pancreatic cancer tissues and normal paracarcinoma tissues were obtained. We found that miR-374a expression was significant up-regulated in pancreatic cancer tissues and cells.

MiR-374a participated in the regulation of cell proliferation, migration and invasion in tumors, and inhibited cell proliferation and invasion of lung adenocarcinoma via binding gene of TGFA [32]. MiR-374a promoted metastasis and EMT by suppressing WIF1, PTEN or Wnt5A expression in breast cancer [19]. MiR-374a served as an oncogene via directly targeting Wnt5a, which regulated cell proliferation, gefitinib-induced apoptosis, migration, invasion and EMT of NSCLC [20]. In this study, we found that overexpression of miR-374a significantly promoted pancreatic cancer PANC-1 cell proliferation.

Tumor migration and metastasis are main cause of high mortality in cancer patients, which plays a crucial role in primary tumor growth and metastasis. miRNAs have been shown have potential therapeutic effects in tumor angiogenesis and metastasis [33,34]. Our research suggested that miR-374a overexpression increased pancreatic cancer cells migration. In epithelial cancers, EMT is regarded as one of the major mechanisms that promotes migration, invasion and metastasis [35,36]. We further revealed that overexpression of miR-374a significantly decreased E-cadherin and ZO-1 protein expression, and increased Vimentin, N-cadherin and β-catenin protein expression, which are the marker in epithelial and mesenchymal cells, respectively. Thus, overexpression of miR-374a promoted cell migration and EMT in the

pancreatic cancer.

In previous study, SRCIN1 was down-regulated in breast cancer [21]. And miR-374a promoted cell proliferation, invasion and migration via binding SRCIN1 in gastric carcinoma [22]. In this study, we observed that SRCIN1 expression was downregulated in pancreatic cancer tissues and cells. However, our data indicated that miR-374a negatively regulated the target gene expression of SRCIN1. Furthermore, overexpression of SRCIN1 inhibited cell proliferation, migration and EMT in pancreatic cancer cell. Therefore, all these results confirmed that miR-374a was directly modulated by SRCIN1, which could promote cell proliferation, migration and EMT in pancreatic cancer.

Taken together, miR-374a significantly promoted cell proliferation, migration and EMT of pancreatic tumor through suppressing SRCIN1. MiR-374a/SRCIN1 axis might be regarded as a novel mechanism and potential therapeutic strategy for pancreatic cancer.

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Not applicable.

Availability of data and materials

The datasets used/or analyzed during the current study are available from the corresponding author on reasonable request.

Authors' contributions

LM was responsible for the study design and the acquisition of data. ZS performed the functional experiments. YZ analyzed the data and revised the manuscript.

Consent for publication

Not applicable.

Competing interests

The authors declare that they no financial conflicts of interest.

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