

Original Article

The inhibiting effects of microRNA-429 on the progression of pancreatic ductal adenocarcinoma cells by inhibiting epithelial mesenchymal transition

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Received December 24, 2020; Accepted February 2, 2021; Epub April 15, 2021; Published April 30, 2021

Abstract: Objective: To research the effects and related mechanism of microRNA (miRNA)-429 in the development of pancreatic ductal adenocarcinoma (PDAC). Methods: The proliferation and invasion ability of cells were evaluated through MTT assay and transwell assay, respectively. The expression of proteins and mRNA were examined by immunofluorescence, western blot, and quantitative real-time polymerase chain reaction (qRT-PCR). Results: The effects and potential mechanism of miR-429 in PDAC cells were explored and evaluated. Our study suggested that miR-429 is closely related with the progression of cancer. Overexpressed miR-429 restricted the mobility and proliferation of PDAC cells by restricting EMT, while down-regulated miR-429 had the opposite effect. These above results implied that miR-429 suppresses the development of PDAC by regulating EMT. Conclusion: MiR-429 inhibits the progression of PDAC cells by regulating EMT. Our study provided a novel potential mechanism for the occurrence of PDAC and laid the foundation for the development of miRNA targeted therapy in patients with PDAC.

Keywords: miRNA-429, epithelial mesenchymal transition, pancreatic ductal adenocarcinoma

Introduction

Pancreatic ductal adenocarcinoma (PDAC) is a frequently occurring malignant tumor with poor prognosis worldwide. Treatment for PDCA is still a challenge for a few patients who meet the resection conditions [1, 2]. The mortality rate of PDAC patients is very high, mainly because as high as 70%-85% of patients were diagnosed with advanced cancer [3]. The median survival time of patients with metastatic PDAC was only 6-12 months [4, 5]. In tertiary medical centers worldwide, the mortality after pancreatectomy is less than 5%, but the incidence of postoperative complications is still as high as 40-50% [6, 7].

Mesenchymal phenotypes are transformed from epithelial cells in the process of epithelial mesenchymal transition (EMT) [8, 9]. EMT is associated with primary tumor infiltration, cell migration, and secondary metastasis in a variety of tumor types (especially the epithelial tumors) [10, 11]. EMT exists in diversified pathological processes, such as wound healing,

renal fibrosis, tumorigenesis, and metastasis. From a physiological point of view, EMT acts as an important wound repair mechanism of tissues and organs through forming fibrous scars in the case of tissue and organ damage. However, the forming of fibrous scars is a pathological state, which can lead to fibrosis and sclerosis of tissues and organs [12]. The associated mechanism has been studied in the fibrosis process of finer tissues such as bronchioles and renal tubules, and there are also many related reports [13, 14]. Recently, EMT has become an important therapeutic target in the treatment of hepatocellular carcinoma by terminating and reversing the progressive liver fibrosis [15, 16].

High-throughput gene expression analysis indicates that microRNA (miRNA or miR) manipulates gene expression through regulating mRNA cleavage [17, 18]. According to previous research, miRNAs have a hand in cell development, proliferation, and apoptosis [19]. An imbalanced expression of specific miRNA is found in various tumors, including pancreatic cancer.

Effects of miR-429 on PDAC cells through EMT regulation

MiR-429 was reported to suppress the development of gastric and renal cancer through binding with c-myc or PLGG1 and regulating the transcription of c-myc or PLGG1 [20]. Over-expression of miR-429 increased the expression level of c-myc, which is an evolutionarily conserved transcription regulator with B/HLH/LZ structure. C-myc participates in the regulation of cell proliferation, differentiation, apoptosis, cell cycle, metabolism of biological macromolecules, and malignant transformation of cells. In addition, heterodimer formed by c-myc and Max binds with the E-box structure in the promoter region to regulate the transcription of target genes. C-myc could also regulate gene expression and activity in both positive and negative directions through other ways [21]. MiR-429 restrains the mobility of gastric cancer cells by regulating the expression of c-myc and its downstream genes. In addition, miR-429 suppresses the progression of renal carcinoma cells by reducing the expression level of CHMP5 protein [22, 23]. However, there are few studies on the detailed mechanism of miR-429 in pancreatic cancer till now.

Increasing evidence show that various miRNAs induces the proliferation, metastasis of cancer cells, or promotes the angiogenesis through EMT regulation. In this study, effects of miR-429 on the proliferation and invasion of PDCA Bxpc-3 cells were explored.

Materials and methods

Cell culture and clinical PDAC tissue samples collection

Human pancreatic cancer cell line Bxpc-3 was purchased from American Type Culture Collection Center (Manassas, Virginia, USA) and was kept in Roswell Park Memorial Institute-1640 medium (Invitrogen, Carlsbad, CA, USA) containing 10% fetal bovine serum (GIBCO, Rockville, MD, USA). The cells were cultured in a humid environment containing 5% CO₂ at 37°C. Clinical PDCA tissues were obtained from recruited PDAC patients (n=3) and were further confirmed through immunohistochemistry. Clinical samples collection informed consent was received from all the selected patients and the samples were used only for clinical research. The clinical sample collection was ratified by the Ethics Committee of our hospital. Inclusion criteria: patients with PDAC; exclu-

sion criteria: patients accompanied with other cancers.

Total RNA extraction

Total RNA in the tissue samples was extracted by Recover All™ nucleic acid separation kit (Life Technologies, USA). 10 um thick sample slices were put into 1.5 ml test tubes. Wax in the tissues was dissolved by Xylene and the protein in the tissues was digested by protease. Then, the mixture was filtered through a filter column and the extracted RNA was used for PCR. The collected liquid contained DNA and RNA, and we used DNase to purify the liquid by degrading DNA. Finally, the eluent was put into a filter column to collect the RNA and the content of RNA was examined by NANO DROP 2000. The maximum absorption wavelength of nucleic acid is 260 nm, which can be used to calculate the concentration of nucleic acid sample. The ratio of OD values at 260 nm and 280 nm can be determined to estimate the purity of nucleic acid. Our experimental results showed that the extracted RNA had high content, and 260/280 analysis showed that the undegradable RNA had higher purity and had no DNA contamination.

Cell transfection

MiR-429 mimics, inhibitors, and the negative control (NC) were bought from Gene Pharma (Shanghai, China). MiRNA sequences were transfected into pancreatic cancer cells using Lipofectamine®2000 (Invitrogen, Carlsbad, California, USA) abided by the proposal of the manufacturer.

Cell proliferation determination

The proliferation ability of PDAC cells under different treatments was measured through 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. After being treated as indicated, Bxpc-3 cells were seed into the 96-well plate. After incubation at 37°C for 0 h, 24 h, 48 h or 72 h, cells were mixed with 10 µL MTT (5 mg/mL) (sigma Aldrich, St. Louis, Missouri, USA) for 4 hours. Subsequently, the crystals in the well were dissolved by 100 µL dimethyl sulfoxide (DMSO) (sigma Aldrich, St. Louis, Mo., USA). The optical density (OD) was measured at 490 nm on the Microplate Reader (Bio-Rad, Hercules, CA, USA).

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Colony formation assay

After being treated with miR-429 mimics or inhibitors as indicated, BXPc-3 cells were plated into a 6-well plate (Suzhou Kangning Co., Ltd., China) for 10 days. After being fixed with methanol (Shanghai Bioengineering Co., Ltd., China) for 30 minutes, the colony was dyed with 1.0% crystal violet (Sinopharm group, China) for 20 minutes.

Cell invasion assay

Fifty-thousands BXPc-3 cells were kept in the upper chamber of transwell (Suzhou Kangning Co., Ltd., China), and 0.7 mL DMEM (Gibco, USA) was added in the lower chamber. The cells were kept at 37°C for 24 hours in a moist incubator containing 5% CO₂. After being fixed with methanol for 30 minutes, the cells were stained with 1.0% crystal violet for 20 minutes. Lastly, the cells penetrating the matrix gel were regarded as invasive cells and the number was recorded.

Immunofluorescence staining and antibodies

PDAC tissue was cut into 1 cm × 1 cm × 1 cm slices. After being embedded in paraffin and dewaxed, the slices were rehydrated in alcohol with different concentration gradient. Then, the slide was placed into the solution containing citrate buffer (pH 6.0) and eluted using microwave oven (Sharp, R-331ZX) at 95°C for 20 minutes to remove the antigen. After being cooled at room temperature for 20 minutes, the endogenous peroxidase was quenched in 3% H₂O₂. Then, the slides were incubated with sealant (ZSGB-BIO, ZLI-9022) for 30 minutes at room temperature. Subsequently, the antigen was detected adhering to the Opal protocol continuously. In short, the sections were incubated with a primary antibody in a moist chamber at 37°C for 2 hours. Then, the HRP labeled secondary antibody (GBI Labs, Polink-1 HRP polymer detection kit) and the Tyramide signal amplification (TSA)-fluors (PerkinElmer, Opal) were used for detection. The slides were then heated in citrate buffer (pH 6.0) for 10 minutes at 95°C. Similarly, each antigen was labeled with different fluorescent groups in a continuous manner. After that, DAPI (1:2000) staining was used to observe the nuclei and Prolong Gold Antifade Mountant coverslip (Thermo Fisher, P36934) was used. Multiple antibodies used in this study included anti-N-cadherin (CST, #43248, 1:200), anti-E-cad-

herin (CST, #3169, 1:500), and Anti-vimentin (Abcam, ab92574, 1:400).

Western blot assay

Whole proteins of PDAC cells were extracted using radioimmunoassay (RIPA) buffer containing protease and phosphatase inhibitors (Roche, Basel, Switzerland). The protein concentration was valued using the Bicinchoninic Acid (BCA) protein kit (Thermo Fisher technology, Ma, USA). Then, an equal amount of protein sample was separated on the 10% sodium dodecyl sulfate polyacrylamide gel electrophoresis. After being transferred with the protein, the polyvinylidene fluoride membrane (Roche, Switzerland) was blocked with 5% skim milk at 37°C for 2 hours. After being washed, using Tris buffered Saline Tween, the membrane was combined with primary antibody at 4°C overnight. The following antibodies were used here: anti-N-cadherin (CST, #43248, 1:200, Opal 620), anti-E-cadherin (CST, #3169, 1:500, Opal 650), and anti-vimentin (Abcam, ab92574, 1:400, Opal 570). After being washed three times, the membrane was combined with horseradish peroxidase-conjugated secondary antibody (1:3000, ab205718, Abcam, Cambridge, MA, USA) at 37°C for 2 hours. Lastly, the protein expression was detected using the enhanced chemiluminescence Kit (Pierce, Rockford, IL, USA).

Statistical analysis

Continuous variables were shown with the form of mean ± standard deviation, and were compared through the Student-t test; categorical variables were compared through the Fisher analysis in Graphpad Prism 2.0. All statistical assessments were performed using the Statistical Package for Social Science (SPSS) software (version 15.0, SPSS Inc., Chicago, Illinois). P<0.05 were considered statistically significant.

Results

MiR-429 inhibits the proliferation of pancreatic ductal adenocarcinoma cells

In order to investigate the tumor suppressive effect of miR-429 in PDAC cell lines, function loss or gain of miR-429 was analyzed. BXPc-3 cells were transfected with miR-429 mimics or inhibitor to achieve the overexpression or inhibition of miR-429 (P<0.05, **Figure 1A** and **1B**).

Effects of miR-429 on PDAC cells through EMT regulation

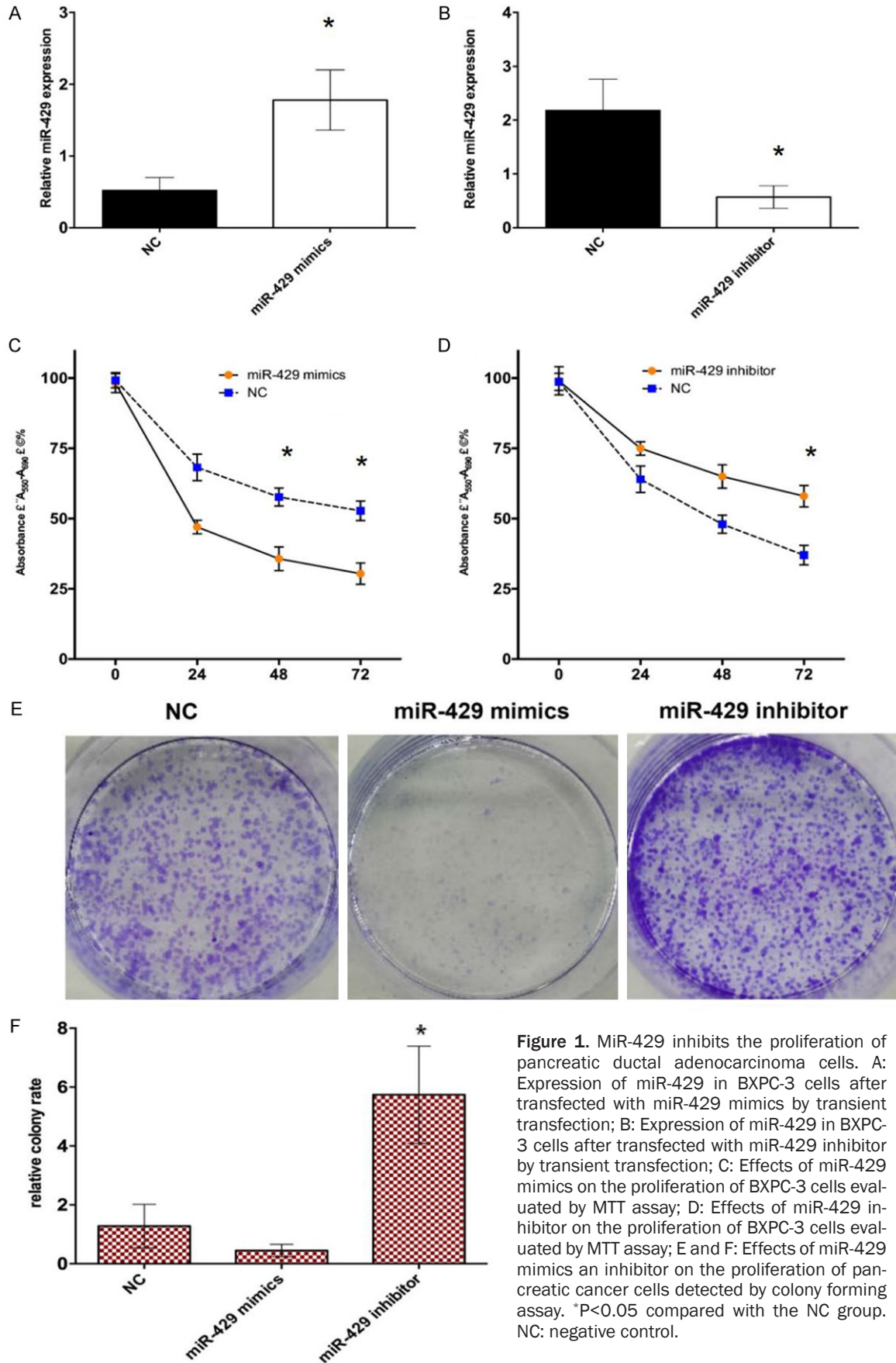


Figure 1. MiR-429 inhibits the proliferation of pancreatic ductal adenocarcinoma cells. **A:** Expression of miR-429 in BXPC-3 cells after transfected with miR-429 mimics by transient transfection; **B:** Expression of miR-429 in BXPC-3 cells after transfected with miR-429 inhibitor by transient transfection; **C:** Effects of miR-429 mimics on the proliferation of BXPC-3 cells evaluated by MTT assay; **D:** Effects of miR-429 inhibitor on the proliferation of BXPC-3 cells evaluated by MTT assay; **E and F:** Effects of miR-429 mimics an inhibitor on the proliferation of pancreatic cancer cells detected by colony forming assay. * $P < 0.05$ compared with the NC group. NC: negative control.

Effects of miR-429 on PDAC cells through EMT regulation

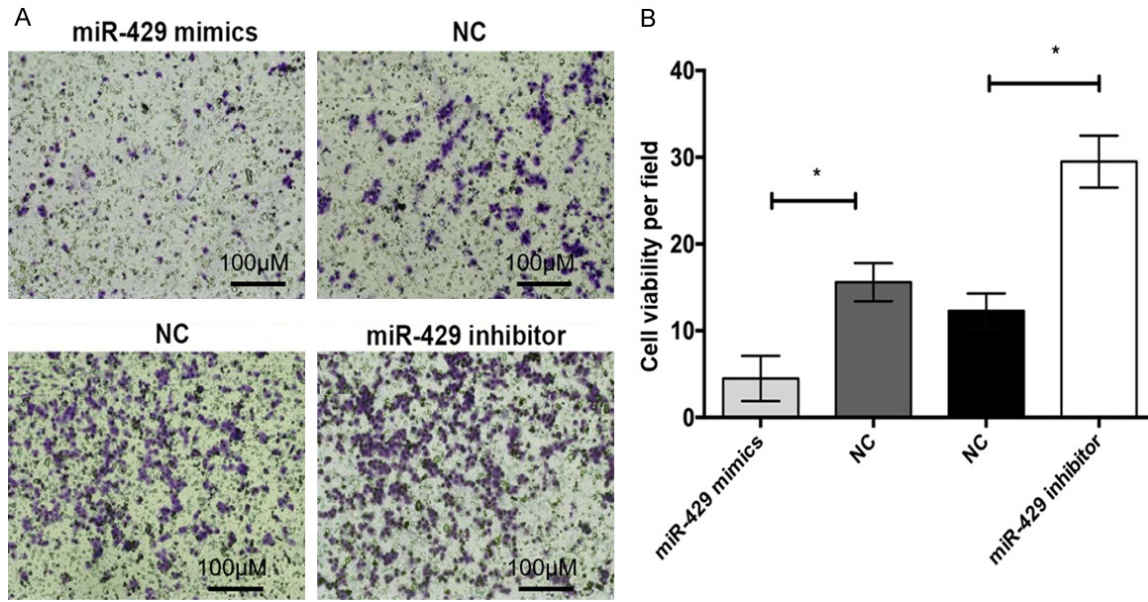


Figure 2. MiR-429 inhibits the invasion of pancreatic ductal adenocarcinoma cells. A: The invasion ability of pancreatic ductal adenocarcinoma cells was evaluated by transwell assay. The cells were transfected with miR-429 mimics and NC (miR-NC) (the first batch of experiments) or miR-429 inhibitor and NC (miR-NC) (the second batch of experiments); the treatment method of miR-NC was consistent in two batch of experiments. The number of transmembrane cells in transwell chamber was photographed under the inverted fluorescence microscope (magnification: 10 ×). B: Corresponding quantitative analysis. Compared with the NC group, * $P < 0.05$.

MTT assay showed that the proliferation of BxPC-3 cells was inhibited by overexpressed miR-429 and was promoted by the inhibition of miR-429 ($P < 0.05$, **Figure 1C** and **1D**). In addition, the colony forming assay also showed that up-regulated miR-429 significantly inhibited cell proliferation ($P < 0.05$, **Figure 1E** and **1F**).

MiR-429 suppresses the invasion of pancreatic ductal adenocarcinoma cells

The transwell assay showed that the invasion of Bxpc-3 cells was largely inhibited by overexpressed miR-429 ($P < 0.05$, **Figure 2A**).

Effects of miR-429 on EMT related factors in pancreatic cancer cells

Potential mechanism of miR-429 on pancreatic ductal adenocarcinoma (PDAC) was analyzed. Firstly, the production of E-cadherin was elevated and the production of Vimentin and N-cadherin was suppressed in BxPC-3 cells by overexpressed miR-429 ($P < 0.05$, **Figure 3A** and **3B**). In contrast, the down-regulated miR-429 in Bxpc-3-miR-429-inhibitor cells resulted in a suppressed production of E-cadherin and elevated production of N-cadherin and Vimentin ($P < 0.05$, **Figure 3A** and **3B**). There were no

significant differences in the expression of E-cadherin, N-cadherin, or Vimentin between Bxpc-3-miR-429-NC group and the BxPC-3 group (**Figure 3A** and **3B**). In addition, expression of E-cadherin was negatively correlated with Vimentin regardless of the expression of miR-429. Highly expressed miR-429 was accompanied with lowly expressed Vimentin, in the form of negative correlation, which was consistent with the results of cell experiment in **Figure 3B**. Expression of E-cadherin and Vimentin in the tissues of PDAC patients detected by immunofluorescence also showed negative correlation. In general, miR-429 was correlated with the expression of E-cadherin and Vimentin (**Figure 3C** and **3D**).

Discussion

Although progress has been achieved in the treatment of most malignant tumors, the treatment for pancreatic cancer is still disappointing till now [24]. Only 15-20% of patients could be diagnosed as respectable timely, which is related to poor survival of pancreatic cancer [25, 26]. Therefore, exploration for the pathogenesis of PDAC could provide a theoretical basis for pancreatic cancer treatment in the future. A

Effects of miR-429 on PDAC cells through EMT regulation

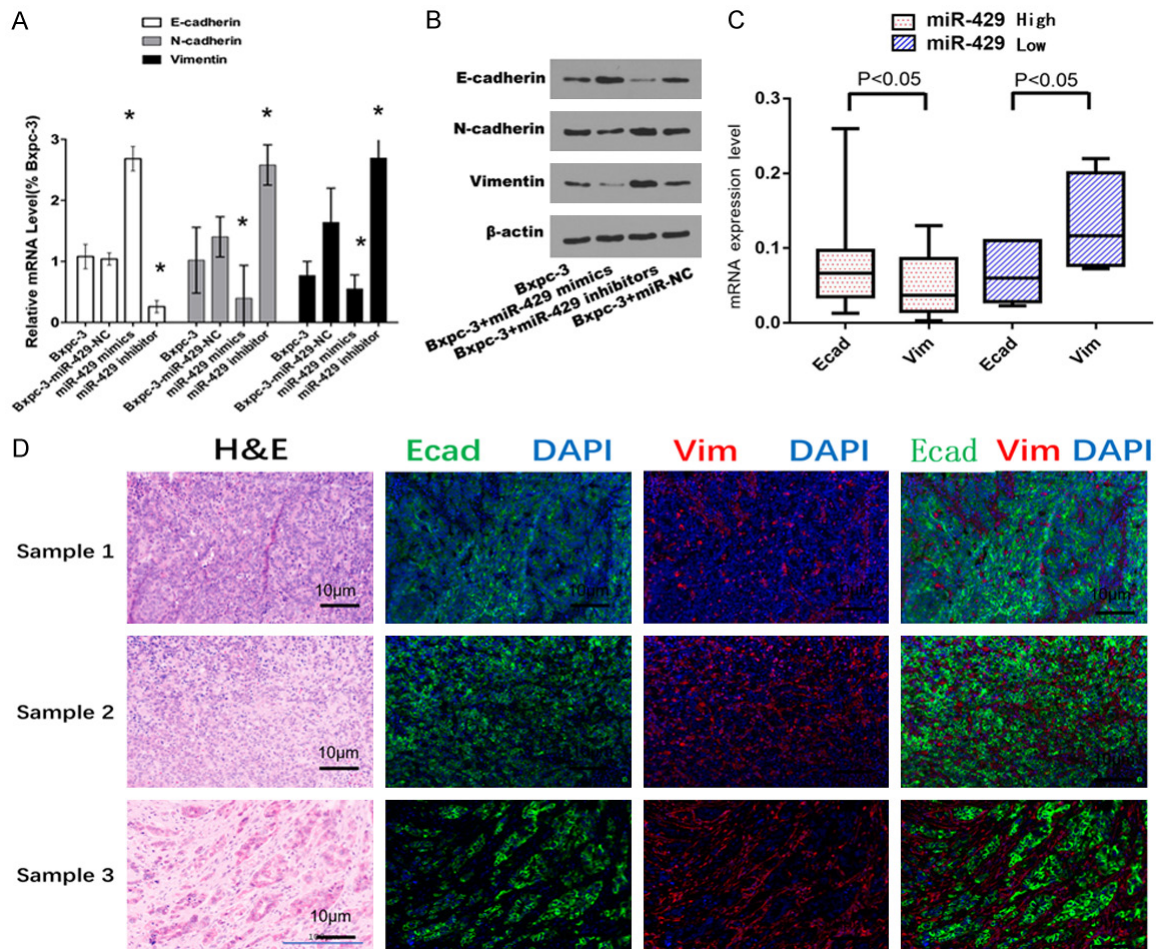


Figure 3. Effects of miR-429 on EMT related factors E/N-cadherin and Vimentin in pancreatic cancer cells and the correlation analysis of miR-429 with E-cadherin and Vimentin expression. A: Effects of miR-429 mimics, miR-429 inhibitor and the control treatment on the mRNA of EMT related factors E-cadherin, N-Cadherin and Vimentin in pancreatic cancer Bxpc-3 cells; B: Effects of miR-429 mimics, miR-429 inhibitor and the control treatment on the protein expressions of EMT related factors E-cadherin, N-Cadherin and Vimentin in pancreatic cancer Bxpc-3 cells; C: The relationship between E-cadherin and Vimentin, and the relationship between miR-429 expression and Vimentin in clinical pancreatic cancer tissues were analyzed; D: The relationship between E-cadherin and Vimentin in clinical pancreatic cancer tissues was detected by immunofluorescence. Immunohistochemical slides of pancreatic cancer tissues were photographed under the inverted fluorescence microscope (magnification: 10 \times). Compared with Bxpc-3-miR-NC group, * $P < 0.05$.

variety of miRNA disorders have been studied in PDAC, which played an inhibitory or carcinogenic role in tumor progression [27].

Abnormal expression of miR-429 is commonly found in ovarian cancer, cervical cancer, breast cancer, and liver cancer [28]. MiR-429 has been demonstrated to be associated with the development, mobility, apoptosis, and drug resistance. MiR-429 has also been regarded as a potential indicator for the diagnosis, treatment, and prognosis of some tumors. At the level of cytology and histology, it can be seen that the expression of several miRNAs accelerates

the EMT process of tumor tissue. Mesenchymal cells may also be the main source of circulating cancer stem cells [29]. Thus, miRNAs could regulate the mobility of cancer cells by affecting the expression of EMT related proteins. In addition, members of the miR-200 family can directly target ZEB1 and ZEB2, and can also be regulated as feedback loops in the process of EMT. Ectopic overexpression of miR-429 induces the transformation of mesenchymal phenotype into epithelial phenotype in metastatic ovarian cancer cells [30]. In addition, miR-429 regulates EMT related markers such as Zinc finger E-box binding protein 2,

Vimentin, SNAI2, and SNAI1 by targeting ONE-CUT2 in colorectal cancer cells [31]. Similarly, our study suggests that miR-429 may play an important role in the progression of cancer. We found that overexpression of miR-429 significantly inhibited the mobility and proliferation of PDAC cells by regulating EMT, while silenced miR-429 played the opposite effect. The expression of miR-429 was significantly decreased in tumor tissues from patients with LA. In addition, the decrease of miR-429 in PDAC is related to the deterioration of clinicopathological features and poor prognosis. Other researchers found that miR-429 had tumor inhibitory effect in some cancers [32]. Based on previous reports and our experimental results, we found that the proliferation and migration of pancreatic ductal carcinoma cells decreased when miR-429 was overexpressed. Therefore, treatment of patients with miR-429 mimics may prolong the survival of patients with pancreatic ductal carcinoma, which has a positive effect on the treatment of pancreatic ductal carcinoma.

This present study still has some limitations. The sample size is small and related mechanisms have not been deeply studied. The relationship between the survival rate of pancreatic cancer patients after treatment and the change of miR-429 expression should be further studied. In addition, we did not detect the direct targets of miR-429. We found that VASH2, HIPK3, MAP2, ERFF1, ZEB1, NR5A2, and ZEB2 were all the direct targets of miR-429 through miRDB analysis. These targets have not been further explored in this study and will need further research in the future.

In conclusion, EMT partially regulates the effects of miR-429 on the proliferation and migration of PDAC cells, which may provide a new research direction for tumorigenesis and provide a theoretical basis for the research of miRNA drug therapy targeting for patients with PDAC.

Disclosure of conflict of interest

None.

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