Epithelial to Mesenchymal Transition (EMT) Regulated by MicroRNA-429 in Pancreatic Ductal Adenocarcinoma (PDAC)

Gang Zheng¹, Zhigang Wang¹, Jie Tang¹, Yin Sun¹, Zhou Hu¹, Huiping Chen¹*

Affiliations:
1 Department of General Surgery, Jingmen First People’s Hospital, Jingmen, Hubei Province, China

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*Corresponding authors:
Correspondence: Huiping Chen, Department of General Surgery, Jingmen First People’s Hospital, Jingmen, Hubei 448000, China
Email: ercolani@126.com

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The data used to support the findings of this study are available from the corresponding author upon request.
Abstract

In this study, we aimed to investigate the role of miRNA-429 in the pathogenesis of pancreatic ductal adenocarcinoma (PDAC) and the potential mechanism of this procedure. Totally 95 consecutive patients with PDAC diagnosed by pathology were retrospectively collected from June 1st, 2015 to August 30th, 2019 in Department of General Surgery, Jingmen First People’s Hospital. The human pancreatic cancer cell line Bxpc-3 and Panc-1 were used and the cell proliferation and migration were detected by MTT assays and Transwell assays, respectively. The quantitative real-time polymerase chain reaction (qRT-PCR) was applied to evaluate the expression in RNA level. Our results showed that overexpression of miRNA-429 could suppress the cell invasion, proliferation and metastasis through regulating the process of EMT in PDAC cell line, while low expression of miRNA-429 had the opposite effects. We demonstrated that miRNA-429 had critical roles in the pathogenesis of PDAC. Clinically, we observed that tumor tissues from patients with PDAC exhibited significantly decreasing in miRNA-429 expression compared with the non-tumor tissues. Additionally, decreased expression of miRNA-429 in tumor tissues of patients with PDAC was associated with poorer prognosis and several clinical-pathological characteristics. In conclusion, miRNA-429 exerted anti-tumor functions in PDAC through the regulation of EMT process. The results of this study would provide a novel insight into tumorigenesis and the basis for the development of miRNA-targeting therapies against PDAC.

Keywords: MicroRNA-429; Invasion and Migration; PDAC.
Introduction

Pancreatic ductal adenocarcinoma (PDAC) is the most lethal forms of human malignancies known to date, with an overall five-year survival rate well below 10% [1-3]. It remained challenging to treat since few patients were diagnosed at early stage for surgical resection. A large proportion of patients were diagnosed at advanced-stage and the median survival for those patients at late stage was 6-12 months [4, 5]. Although mortality rates following pancreatectomy are now less than 5% in high-volume tertiary referral centers, morbidity following pancreatectomy is still common with rates estimated as high as 40–50% [6, 7].

Epithelial-mesenchymal transition (EMT) is a highly regulated process by which epithelial cells transform into a mesenchymal cell phenotype. EMT is associated with several processes including primary tumor invasion, cell migration and secondary metastasis formation in a variety types of cancers, particularly in those of epithelial origin [8-11]. In addition, EMT is also present in a variety of pathological processes, such as wound healing, renal fibrosis, tumorigenesis and tumor metastasis. Under physiological conditions, EMT is an important wound repair mechanism for tissues and organs. After tissues were damaged, the fibrous scars are formed by EMT to achieve wound repair. However, the existence of this process is also a pathological state, which can cause tissue and organ fibrosis and sclerosis [12, 13]. The mechanism has been studied earlier in the fibrosis of tubular tissues such as bronchioles and renal tubules [14-16]. Particularly, researches had reported that EMT had become an important therapeutic target on suppressing the development of hepatocellular carcinoma [17, 18].

Recent improvements in high-throughput gene expression detection and analysis have revealed that microRNAs (miRNAs) are important and manipulate local or global gene expression. Among various types of miRNAs, the endogenous miRNAs are involved in cell development, proliferation and apoptosis in normal physiological processes [19-21]. Under pathological conditions, the occurrence of many kinds of
tumors is accompanied by the imbalance of specific miRNA expression. Previous studies had shown that miRNA-429 had the capability to inhibit tumor development by binding to c-myc and PLGG1 in gastric and renal cell carcinoma[22, 23]. Researchers have also demonstrated that miRNA-429 played a tumor suppressing role in colorectal cancer[24]. Nevertheless, there was no study which had investigated the detailed mechanisms of miRNA-429 in PDAC previously.

Increasing evidence had shown that various microRNAs were closely related with EMT induced cancer cell proliferation, metastasis and angiogenesis[25-27]. Studies elucidated the role of miRNA-429 in EMT and it could make c-myc, c-myb, MYCN, Cyclin A and CDK8 as potential targets to further regulate the EMT process[28][29]. In the present study, we used bioinformatics analysis to identify miRNA-429 enhancement in PDAC and we investigated the molecular mechanism of miRNA-429 regulating the EMT in the development of pancreatic cancer.

Patients and methods

1. Study design and participants

The study included 95 consecutive patients with PDAC diagnosed by pathology were retrospectively collected from June 1st, 2015 to August 30th, 2019 in Department of General Surgery, Jingmen First People’s Hospital. The study was approved by the Regional Ethical Review Board for Jingmen First People’s Hospital. The number of the local ethical board review is JMU04138. Patients were treated according to the Declaration of Helsinki's ethical principles for medical research involving human subjects. All patients provided an informed written consent prior to study entry. Patients were required to meet the following inclusion criteria: Participants were aged 18 to 80 years; Eastern Cooperative Oncology Group performance status (ECOG-PS)[30]: 0-1; the primary treatment procedure was surgical resection; histologically or cytologically confirmed PDAC. The non-tumorous and tumorous samples were collected during the surgical procedures. The and the non-tumorous tissues were identified as tissues more than 1cm from the tumor tissues. No prior chemotherapy or immunotherapy was allowed for those
patients. Patients were excluded if they had a concurrent malignancy other than PDAC, a serious, uncontrollable medical condition, or a psychiatric disorder that would limit ability to comply with study requirements.

2. Cell Cultures

Human pancreatic cancer cell lines Bxpc-3 and Panc-1 were purchased from the American Type Culture Collection (Manassas, VA, USA). All the cells were maintained in Roswell Park Memorial Institute-1640 (RPMI-1640) medium (Invitrogen, Carlsbad, CA, USA) which contained 10% fetal bovine serum (FBS) (Gibco, Rockville, MD, USA) in a humidified atmosphere at 37°C with 5% CO2.

3. Total RNA extraction

We used Recover All™ Total Nucleic Acid Isolation Kit to extract the total RNA from all the tissues according to the manufacturer’s instructions. The maximum absorption wavelength of nucleic acid is 260nm, which can be used to calculate the concentration of nucleic acid sample. The ratio of OD value at 260 nm and 280 nm can be determined to estimate the purity of nucleic acid. The extracted RNA in this study was higher in content, and 260/280 analysis indicated un-degraded RNA, with higher purity, without pollution of DNA.

4. qRT-PCR.

QRT-PCR was performed by using the Light Cycler 480 (Roche) and the Fast SYBR Green Master Mix (Applied Biosystems). For the mature miRNA analyses, cDNA was generated and qRT-PCR was performed using the Exiqon Universal cDNA Synthesis Kit, SYBR Green Master Mix, and commercially available primers (Exiqon). mRNA and miRNA expression were normalized using detection of GAPDH (Applied Biosystems), respectively. Results are represented as fold induction using the \( \Delta \Delta \text{Ct} \) method with the control set to 1 as described before[31]. The MicroRNA-429, MicroRNA-429 mimic and MicroRNA-429 inhibitor primers were as follows:
MicroRNA-429: forward: 5′ -CTCGAGGCTCATAGTGAACACAGC-3′ ;
reverse: 5′ -GAGCCCGAGTATCCTTGGTGTGG-3′ ;

MicroRNA-429 mimic: forward: 5′ -CTCGGCCGGCGATTTCCCGTTTG-3′ ,
reverse: 5′ -TAGCGGCCGCTAAAGGGCAAACAC-3′ ;

MicroRNA-429 inhibitor: forward: 5′ -CTCGAGGGGATGATGTGGTTT-3′ ;
reverse: 5′ -ACACTCGATGTATCTAAGAATATAAGTC-3′ .

5. Cell Transfection

To induce miRNA-429 overexpression or silencing, the cells were transfected with negative control miRNA (NC miRNA), miRNA-mimics and miRNA-inhibitor (Gene Pharma Co., Ltd., Shanghai, China) using Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA) following the manufacturer’s instructions. HiPerFect Transfection Reagents (Invitrogen, Carlsbad, CA, USA) were used for miRNA overexpression and silence. Briefly, the materials used to transfect the cells (miRNA-429 mimic and miRNA-429 inhibitor) were diluted with serum-free DMEM. Lipofectamine 2000 was also diluted with serum-free DMEM. The diluted Lipofectamine 2000 was added into the diluted plasmid, or miRNA mimic, or inhibitor, and incubated for 20 min at room temperature, and subsequently added to the Bxpc-3 and Panc-1 cells at ~70% confluence in a 6 - well plate. Subsequently, the cells were incubated at 37°C in an atmosphere of 5% CO₂ for 6 h. Following incubation, the medium in each well was replaced by the DMEM supplemented with 10% FBS, and cultured for 24 h at 37°C prior to performance of the following assays.

6. Cell Proliferation Assays

MTT assays were performed to measure the proliferation ability of Bxpc-3 and Panc-1 cells with different treatment. After transfected with miRNA-429 mimics or
inhibitor, PDAC cells were harvested and plated into 96-well plate. After incubated for 0 h, 24 h, 48 h or 72 h at 37°C, MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-di-phenyltetrazoliumbromide)(10 μL, 5 mg/mL)( Sigma-Aldrich, St. Louis, MO, USA) was added into each well, followed by an incubation for another 4 h. Subsequently, 100 μL dimethyl sulfoxide (DMSO) (Sigma-Aldrich, St. Louis, MO, USA) was added to solubilize the crystals. The optical density (OD) 490 nm was measured with a microplate reader (Bio-Rad, Hercules, CA, USA).

7. Colony formation assay

After the cancer cells were transfected with miRNA-429 mimic or inhibitor, they were cultured in a 6-well plate for 10 days. The colonies were fixed with methanol for 30 min and stained with 1.0% crystal violet for 20 min. Differences in colony number and size were evaluated by a two-tailed one-sample t-test to test for variability between individual samples.

8. Scratch-healing migration assay

Briefly, cells were seeded at 5 × 10^4 cells/well in 24-well plates and cultured for 24 h. Wounds were created using a 10-μl pipette tip. Wound healing was assessed after 24 h. We randomly selected 5 locations for assessment and photographing. Images were obtained with a Zeiss Ax overt 200 microscope.

9. Cell invasion assay

Briefly, 5 × 10^4 cells were added into the upper chamber of a Transwell and 0.7 ml DMEM was added to the lower chamber. Cells were cultured for 24 h at 37 °C in a humidified incubator with 5% CO2. After treatment, the cells were fixed with methanol for 30 min and stained with 1.0% crystal violet for 20 min. The number of invasive cells penetrating the Matrigel was recorded.

10. Immunofluorescence staining and antibody
For Histo-Cytometry (Multiplex Quantitative Tissue Imaging Analysis), slides loaded with tissue sample were deparaffinized in xylene and rehydrated in a series of graded alcohols. Antigen retrievals were performed in citrate buffer (pH6) with a microwave (Sharp, R-331ZX) for 20 min at 95°C followed by a 20 min cool down at room temperature. After the quenching of endogenous peroxidase in 3% H2O2, slides were incubated with blocking reagent (ZSGB-BIO, ZLI-9022) for 30 min at room temperature. Antigens were then successively detected using the Opal protocol. Briefly, each primary antibody was incubated for 2 h in a humidified chamber at 37°C, followed by detection using the HRP-conjugated secondary antibody (GBI Labs, Polink-1 HRP polymer detection kit) and TSA-fluors (PerkinElmer, Opal 7-color IHC Kit, NEL797001KT, 1:100, 20–60 s), after which the primary and secondary antibodies were thoroughly eliminated by heating the slides in citrate buffer (pH 6.0) for 10-min at 95°C using microwave. In a serial fashion, each antigen was labeled by distinct fluorophores. Nuclei were subsequently visualized with DAPI (1:2000), and the slides were cover slipped using Prolong Gold Antifade Mountant (ThermoFisher, P36934). Multiplex antibody panels applied in this study are: panel 1: N-Cadherin (CST, #43248, 1:200, Opal 620), E-Cadherin (CST, #3169, 1:500, Opal 650), Anti-Vimentin (Abcam, ab92574, 1:400, Opal 570). All the antibodies were diluted by PBS.

11. Statistical methods.

Continuous variables were expressed as mean ± SD (standard deviation) and compared using a two-tailed unpaired Student’s t test; categorical variables were compared using χ2 or Fisher analysis. Life-table estimates of survival time were calculated according to the Kaplan and Meier methodology [32]. The Greenwood formula was used for the standard deviation. A confidence interval which did not include the value 1 indicated statistical significance at the 5% level. All statistical evaluations were carried out using SPSS software (Statistical Package for the Social
Science, version 15.0, SPSS Inc, Chicago, IL). A value of p<0.05 was considered to be statistically significant in all the analyses.
Results

1. Patients’ characteristics.

Among the 95 patients enrolled in this study, 55 patients received adjuvant chemotherapy after the operations. In these patients, 21 patients received the treatment of FOLFIRINOX and 34 patients received nab-paclitaxel combined with gemcitabine. Patients were received blood routine tests at multiple timepoints. Characteristics of all patients are detailed in Table 1. Among these variables, TNM staging system and tumor vascular invasion were significantly related with the expression of miRNA-429.

2. MiRNA-429 downregulation was associated with poor prognosis in patients with PDAC.

To elucidate the prognostic value of miRNA-429 in PDAC, we have examined the expressions of miRNA-429 in both tumor and non-tumor tissues. The qRT-PCR results showed that miRNA-429 expressions were notably reduced in PDAC tissues in comparison with normal tissues in the same patients (Figure 1A). In addition, we further investigated the clinical significance of miRNA-429 in predicting prognosis in patients with PDAC. The mean miRNA-429 expression was used as the cut-off value to divided the PDAC patients into miRNA-429 high expressing group and low expressing group. Further analysis showed that low miRNA-429 expression was associated with several clinicopathologic features in PDAC patients. Moreover, we performed the Kaplan-Meier analysis and found that the low miRNA-429 expressions were prominently associated with poorer overall survival (OS) and progressive free survival (PFS) in PDAC patients (Figure 1B, C).

3. MiRNA-429 suppressed the cell proliferation in pancreatic cancer cell line

To further investigate the tumor suppressive roles of miRNA-429 in PDAC cancer cell line, MTT assay was carried out. Briefly, the overexpression and inhibition of miRNA-429 were obtained by transiently transfecting miRNA-429
mimics and inhibitor into Bxpc-3 and Panc-1 cells (Figure 2A, 2B). The MTT assays revealed that miRNA-429 overexpression dramatically inhibited Bxpc-3 and Panc-1 cell proliferation (Figure 2C, 2D). On the contrary, we found that inhibition of miRNA-429 expression could increase the cell proliferation (Figure 2A-D). We then performed colony formation assays and found that overexpression of miRNA-429 significantly suppress the colony formation of pancreatic cancer cell line (Fig. 3A, 3B).

4. MiRNA-429 inhibited cell invasion and migration in pancreatic cancer cell line.

In this study, transwell assay was carried out to determine the invasion and migration abilities of PDAC cancer cells with different transfections. The results showed that miRNA-429 upregulation in both Bxpc-3 and Panc-1 dramatically suppressed the invasion and migration capabilities of both Bxpc-3 and Panc-1 were cells (Figure 4A and 4B).

5. MiRNA-429 regulated the activity of pancreatic cells by EMT and associated with the expression of factors related with EMT

We further investigated the underlying mechanisms of inhibitory effect mediated by miRA-429 in PDAC progression. Firstly, both Bxpc-3 and Panc-1-miRNA-429-inhibitor cells were associated with increased expression of E-cadherin and decreased expression of N-cadherin and vimentin (Fig. 5A, B). By contrast, both Bxpc-3 and Panc-1-miRNA-429-mimic cells resulted in downregulation of E-cadherin and upregulation of N-cadherin and vimentin (P<0.05; Fig. 5 A, 5 B). Bxpc-3 and Bxpc-3-miRNA-429 - NC, Panc-1 and Panc-1-miRNA-429 - NC cells exhibited no significant differences in the expression of E-cadherin, N-cadherin or vimentin (Fig. 5 A, B). Secondly, we performed immunofluorescence to examine the EMT factors expressions in PDAC tissues. The E-cadherin expression was significantly higher in the low miRNA-429 expression
Discussion

Despite of the huge improvement in the treatment of most malignant tumors and the enhancement of understanding of cancer pathogenesis during these decades, the outcomes of patients with pancreatic cancer remained dismal and unsatisfied. One of the reasons for extremely poor survival outcome in pancreatic cancer was that only 15–20% of all patients with pancreatic cancer were diagnosed early enough to be resectable[33, 34]. Therefore, developments in PDAC have heightened the need for more research on the pathogenesis of PDAC to provide theoretical basis for further PDAC therapies. During these years, various miRNAs has been focused to be identified as prognostic biomarkers and several detailed mechanisms have been investigated in PDAC. Several miRNAs functioned as either a suppressor or an oncogene in tumor progression[35-37].

At the cytological and histological levels, studies have demonstrated that the expression of several miRNAs had the ability to accelerate the EMT process of tumor tissues. Moreover, based on the previous studies, the mesenchymal cells were the main source of circulating cancer stem cells, which had deeply interaction with the tumor cells[38, 39]. Meanwhile, the overexpression of several specific miRNAs could increase the circulating tumor cells in patients and accelerate the tumor metastasis[40]. Based on this, we performed validation of complementary study for previous cytology and zoology experiments with respect to the relation of mesenchymal cells with miRNA-429 expression in the tumor tissue of patients and the potential mechanism of miRNA-429 on regulating EMT.
In this study, we evaluated the expression level of miRNA-429 in PDAC cells and human PDAC tissues and the functional effects and potential mechanism of miRNA-429 in PDAC were investigated. Our results showed that overexpression of miRNA-429 could suppress the cell invasion, proliferation and metastasis through regulating the process of EMT in PDAC cell line, while low expression of miRNA-429 had the opposite effects. We demonstrated that miRNA-429 had critical roles in the pathogenesis of PDAC. Clinically, we observed that tumor tissues from patients with PDAC exhibited significantly decreasing in miRNA-429 expression compared with the non-tumor tissues. Additionally, decreased expression of miRNA-429 in tumor tissues of patients with PDAC was associated with poorer prognosis and several clinical-pathological characteristics.

There were several limitations of this study: on the one hand, this is a retrospective study with small sample size. On the other hand, the relationship between patients survival and the change of miRNA-429 levels after treatment apart from pretreatment can be investigated in future studies.

In conclusion, miRNA-429 exerted anti-tumor functions in PDAC through the regulation of EMT process. The results of this study would provide a novel insight into tumorigenesis and the basis for the development of miRNA-targeting therapies against PDAC.

**Author contributions:** Gang Zheng and Huiping Chen designed the research; Zhigang Wang Jie Tang Yin Sun and Zhou Hu conducted acquisition of data; Gang Zheng and Huiping Chen performed research and statistical analysis; Gang Zheng and Huiping Chen wrote the paper; Gang Zheng and Huiping Chen conducted a critical revision of the manuscript.
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Figure legends:

Figure 1. miR-429 Downregulation Was Associated with Poor Prognosis in patients with PDAC. A: comparison of miR-429 expression in mRNA level; B, C: high miR-429 was associated with favorable overall survival(B) and progressive free survival(C).

Figure 2. effect of miR-429 on cell proliferation. A, B: overexpression or inhibition of miR-429 was obtained by transiently transfecting miR-429 mimics or inhibitor into Bxpc-3 and Panc-1 cells; C, D: the MTT assays revealed that miR-429 overexpression dramatically inhibited Bxpc-3 and Panc-1 cell proliferation.

Figure 3. Colony formation assays on Bxpc-3 and Panc-1 cell lines. A, B: miR-429 inhibition significantly increased the rate of cell proliferation.

Figure 4. effect of miR-429 on cell invasion and migration. A: miR-429 Inhibited cell migration in pancreatic cell line: miR-429 mimics(a) vs. NC(b) and NC(c) vs. miR-429 inhibitor(d); B: miR-429 Inhibitor significant suppress the cell invasion. *P<0.05.

Figure 5. expression of factors related with EMT. A, B: factors associated with EMT differed in various groups according to miR-429 Regulation; C: Immunofluorescence showed that different expression of E-cadherin and vimentin between the groups of miR-429 high and low expression.
Table 1. Demographics and clinical characteristics of all patients.

| Variable                        | MiR-429 low (N = 30) | MiR-429 high (N = 65) | P values |
|---------------------------------|----------------------|-----------------------|----------|
| Age                             | 61.3 ± 10.4          | 63.4 ± 11.4           | 0.839    |
| Gender                          |                      |                       |          |
| Female                          | 11                   | 24                    | 0.639    |
| Male                            | 19                   | 41                    |          |
| ECOG-PS                          |                      |                       | 0.329    |
| 1                               | 27                   | 57                    |          |
| 2                               | 3                    | 8                     |          |
| TNM staging                     |                      |                       | 0.003    |
| I-II                            | 18                   | 60                    |          |
| III                             | 12                   | 5                     |          |
| Adjuvant chemotherapy           |                      |                       | 0.178    |
| FOLFIRINOX                      | 9                    | 12                    | 0.383    |
| nab-paclitaxel combined with gemcitabine | 14  | 20        |          |
| Estimated blood loss (ml)       | 1198.6 ± 863.3       | 1253.4 ± 943.3        | 0.352    |
| Tumor size (cm)                 | 2.58 ± 3.24          | 2.49 ± 4.56           | 0.426    |
| Neural Invasion                 |                      |                       |          |
| yes                             | 15                   | 36                    | 0.665    |
| no                              | 15                   | 29                    |          |
| Vascular invasion               |                      |                       | 0.013    |
| yes                             | 18                   | 12                    |          |
| no                              | 12                   | 53                    |          |
| CA-199                          |                      |                       | 0.249    |
| > 12000                         | 20                   | 38                    |          |
| < 12000                         | 10                   | 27                    |          |