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MiR-19 enhances pancreatic cancer progression by targeting PTEN through PI3K/AKT signaling pathway

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Abstract. – OBJECTIVE: Abnormal expression of micro ribonucleic acids (miRNAs) has become an important marker of cancer. However, the exact molecular mechanisms of miRNAs were not very clear. Here, we decided to investigate the miR-19 effect and molecular mechanism on pancreatic cancer, which was blank until now.

PATIENTS AND METHODS: Quantitative real-time polymerase chain reaction (qRT-PCR) analysis was applied for testing miR-19 and gene of phosphate and tensin homolog deleted on chromosome ten (PTEN) expression. Western blot was used for detecting the protein expression. Methyl thiazolyl tetrazolium (MTT) assay and transwell assay were carried out to measure cell proliferation, invasion, and migration.

RESULTS: We showed that miR-19 expression was increased in cancerous tissues and was associated with the survival of patients, tumor node metastasis (TNM) stage, tumor size, and lymph node metastasis. MiR-19 mimic enhanced cell proliferation, invasion, and migration, while suppressing miR-19 cell progression was suppressed. With the help of TargetScanHuman and luciferase reporter assay, we verified PTEN as a specific target of miR-19. Moreover, PTEN expression was reduced by miR-19 mimic and was increased by miR-19 inhibitor. We next found that PTEN was elevated in cancerous tissues and its expression was negatively correlated with miR-19 expression. Furthermore, miR-19 regulated cell progression via activating phosphatidylinositol 3-hydroxy kinase/protein kinase B (PI3K/AKT) signaling pathway.

CONCLUSIONS: We demonstrated that miR-19 facilitated cell progression through modulating PI3K/AKT signaling pathway by targeting PTEN, which provided a potential therapeutic target for pancreatic cancer patients.

Key Words:
MiR-19, Pancreatic cancer, PTEN, Progression, PI3K/AKT.

Introduction

Pancreatic cancer is a malignant tumor of the digestive tract, which is difficult to diagnose and treat, and about 90% of the patients are pancreatic ductal adenocarcinoma (PDAC) originating from the epithelium of the gland. Although enormous efforts have made to improve the diagnosis and treatment, it remains a deadly malignancy, with an overall five-year survival rate of only 8%[1]. Therefore, a potential strategy to prevent and treat metastatic tumors is urgently needed. Epithelial-mesenchymal transformation (EMT) is a cellular process that drives epithelial cells to achieve mobility and invasiveness[2,3]. Although evidence point to a variety of different views, several researchers still believe that EMT is the key to cancer metastasis[4]. Moreover, it was reported that up-regulation of PI3K reduced the activation of phosphatidylinositol 3-hydroxy kinase/protein kinase B (PI3K/AKT), which played an important role in cell proliferation, migration, and tumorigenesis[5].

Micro ribonucleic acids (miRNAs) was reported to play an important role in most malignancy studies due to its regulatory effect on tumor progression as an oncogene or a suppressor[6,7]. Many researches have elucidated that miRNAs involved in the tumorigenesis of PDAC. MiR-874 suppressed PDAC progression by inhibiting polo-like kinase 1 (PLK1)[8]. However, increasing miR-361-3p enhanced the migration and invasion of PDAC[9]. Here, our research was focused on miR-19. It was reported that miR-19 have a close connection with many various cancers, such as breast cancer[10], lung cancer[11], gastric cancer[12], and colorectal cancer[13]. However, the role of miR-19 and its exact molecular mechanism in PDAC remains uncertain as far as we know, which need us to explore it further.

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Phosphate and tensin homolog deleted on chromosome ten (PTEN), named MMAC1/TEP1, and its expression was decreased in tumors. It was acted as a tumor suppressor by three laboratories[14,15]. Subsequent studies have verified that PTEN is a negative regulator of cell growth and the survival signaling pathway, such as PI3K/AKT signaling pathway[16]. Investigations established that PTEN played an important effect on growth and survival of tumors[17], including prostate cancer[18], colon cancer[19], and ovarian carcinoma[19]. However, its role in PDAC progression was not studied now and whether it acted as a target of miR-19 in regulating PDAC progression was not reported too.

In our work, we explored the role of miR-19 and its exact mechanism in modulating PDAC cell proliferation, invasion, and migration. We observed that miR-19 played a promoting effect on PDAC progression while PTEN has done the opposite effect. We also verified PTEN as a specific target of miR-19 and miR-19 enhanced cell progression *via* regulating PI3K/AKT signaling, suggesting that miR-19/PETN/PI3K/AKT signaling pathway is involved in regulating PDAC progression and might provide a target for treating PDAC.

Patients and Methods

Cell Lines and Cell Transfection

PDAC cell lines SW1990, BxPC-3, AsPC-1, PANC-1, and control HPNE cells were obtained from the American Type Culture Collection (ATCC, Manassas, VA, USA). We cultured the cells routinely in Roswell Park Memorial Institute-1640 (RPMI-1640, Hyclone, South Logan, UT, USA) medium, with 10% fetal bovine serum (FBS, Hyclone, South Logan, UT, USA), penicillin (100 U/mL) and streptomycin (100 μg/mL) at 37°C with 5% CO_2.

For cells transfection, we put the cells in 6-well plates, and then, with the help of Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA), we transfected the miR-19 mimic, inhibitor or plasmids into the cells. Next, we incubated them for 48 h with 5% CO_2 at 37°C. Finally, the quantitative real-time polymerase chain reaction (qRT-PCR) or Western blot was applied for evaluating the transfection efficiency.

Patients and Specimens

PDAC and normal tissues were acquired from 61 patients who underwent pancreatic resection in Fujian Provincial Hospital from February 2011 to February 2017. The specimens were put into liquid nitrogen instantly when the tumor tissues were removed and then stored in -80°C refrigerator for mRNA and protein detection. Table I exhibited the clinical characteristics of patients. The Ethics Committee of Fujian Provincial Hospital approved this project and all patients signed the informed consent.

Transwell Assay

As described previously, the transwell assays were applied for measuring cell invasion and migration. The chamber with 8 μm pore size inserts coated with or without Matrigel was used for assaying the motility and invasiveness of cells. The upper chamber was filled with serum-free medium with cells (2×10^5) suspended and incubated for 24 h. Then, 200 μL medium containing 10% FBS were seeded in the lower chamber. After incubation for 48 h, the migrated or invaded cells in the lower chamber from the upper chamber were fixed with methanol for 10 min and stained with 0.1% crystal violet for another 15 min. The non-migrated or non-invaded cells in the upper surface were removed by a cotton swab. The migrated or invaded cells were photographed using a microscope. We chose ten random fields to count the cells at 100× magnification.

Methyl Thiazolyl Tetrazolium (MTT) Assay

As described previously, MTT (Beyotime, Shanghai, China) assay was applied for measuring cell viability. We seeded 4×10^4 cells in each well of 96-well plates and incubated at 37°C with 5% CO_2 for 4 days. We added MTT (10 μL) into each well every day and then incubated them for another 4 h. The media were removed after the incubation; then, we added 150 μL dimethyl sulfoxide (DMSO) (Sigma-Aldrich, St. Louis, MO, USA) into the wells. The cell viability was calculated by measuring the optical density (OD) at 490 nm.

Luciferase Reporter Assay

For the luciferase reporter assay, the 3′-untranslated region (3′-UTR) of wild-PTEN (WT) and mutant-PTEN (MUT) were first cloned into pmirGLO-Report vector. Then, miR-19 mimic and Luciferase constructs were co-transfected into PANC-1 cells following the manufacturer's protocol. After transfection, the cells were harvested 24 h, and the Dual Luciferase Reporter Assay System (Promega, Madison, WI, USA) was applied for measuring luciferase activity.

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

TRIzol (Invitrogen, Carlsbad, CA, USA) reagent was used for extracting or isolating total RNA from cell lines or tumor samples following the manufacturer's instructions and the first strand complementary deoxyribonucleic acid (cDNA) was synthesized using the ReverTraAce qPCR RT Kit (Thermo Fisher Scientific, Waltham, MA, USA) according to the manufacturer's protocol. QRT-PCR was performed with the help of the 7500 FAST RT-PCR System. The $2^{-\Delta\Delta T}$ method was carried out to calculate and quantify the miR-19 relative expression after standardized the control expression. The endogenous controls were served by U6 and glyceraldehyde 3-phosphate dehydrogenase (GAPDH), respectively. The primer sequences were shown in Table II.

Western Blotting

Radioimmunoprecipitation assay (RIPA) buffer containing 1% phenylmethanesulfonyl fluoride (PMSF; Beyotime, Shanghai, China) was used for extracting or isolating total proteins from PDAC cells or tissues. Protein specimens (50 μg) from different groups were added onto 10% dodecyl sulfate sodium-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to nitrocellulose filter (NC) membranes under an electric field at 90 V for 30 min. These membranes were incubated with 5% milk for 2 h at temperature before a primary antibody was added to incubate them at 4°C overnight and subsequently with secondary antibodies (Santa Cruz Biotechnology, Santa Cruz, CA, USA) at temperature for 2 h. The enhanced chemiluminescence kit (ECL, Millipore, Billerica, MA, USA) was used to detect the signals. The target protein/β-actin level was used to evaluate the target protein relative expression.

Immunohistochemistry Analysis

The HCC tissues were fixed with 4% paraformaldehyde in phosphate buffer saline (PBS) for at least 12 h. Paraffin sections (6 μm) were obtained and then incubated with 3% H_2O_2 in PBS. These sections were blocked with 5% goat milk for 2 h at temperature before a primary antibody anti-PTEN was added to incubate them at 4°C for 48 h and subsequently with biotinylated goat anti-rabbit IgG secondary antibodies (Santa Cruz, Santa Cruz, CA, USA) at temperature for 2 h. The diaminobenzidine (DAB) mixture was used to stain the sections. Then, a graded alcohol series was used to stain sections dehydrated, xylene to clear and

Table I. The clinicopathological relevance analysis of miR-19 expression in PDAC patients.

Characteristics	Cases	miR-19		*p*-value
		High	Low	
Age (years)				0.729
≥ 65	36	20	16	
< 65	25	15	10	
Gender				0.240
Male	30	19	11	
Female	31	15	16	
CA199				0.133
≥ 100	38	24	14	
< 100	23	10	13	
Tumor size				0.010*
< 2.5 cm	45	33	12	
≥ 2.5 cm	16	6	10	
Differentiation grade				0.299
Well-moderately	43	23	20	
Poorly	18	7	11	
TNM stage				0.021*
I-II	20	8	12	
III-IV	41	29	12	
Lymph node metastasis				0.002*
Absence	39	27	12	
Presence	22	6	16	

Statistical analyses were performed by the χ^2-test. *p<0.05 was considered significant.

Table I. Primer sequences for RT-qPCR.

Genes	Sequence
miR-19	F:5′-ACCTGTGCAAATCCATG-3′ R: 5′-TGCGTGTCGTGGAGTC-3′
U6	F: 5'-CGCTTCGGCAGCACATATACTAAAATTGGAAC-3' R:5'-GCTTCACGAATTTGCGTGTCATCCTTGC-3’
PTEN	F: 5'-AAAACAGTAGAGGAGCCGTCAAATC-3' R: 5'-TTCATCAAAAGGTTCATTCTCTGGA-3'
GAPDH	F: 5'-GCTGCCCAACGCACCGAATA-3' R: 5'-GAGTCAACGGATTTGGTCGT-3'

neutral balsam to the coverslip. Finally, the protein density of per section was determined by the Image Pros Plus 5.0 software (Media Cybernetics, Silver Springs, MD, USA).

Xenograft Tumor Formation Assay

Shanghai Lab Animal Research Center (Shanghai, China) provided us the nude mice (3-5 weeks old). The animal experiments were approved by the Animal Care and Use Committee of Fujian Provincial Hospital and conducted according to the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health. The nude mice's right flank was injected with 5×10^6 transfected cells with pre-miR-19 plasmid or negative control. We observed the tumor volume every 4 days. After 28, the mice were sacrificed by CO_2 asphyxiation and tumors were used for further study.

Statistical Analysis

All the experiments were carried out three times. Statistical Product and Service Solutions (SPSS) 17.0 statistical software (SPSS, Chicago, IL, USA) was applied to process the data. The data was displayed as mean ± SD (standard deviation). The Student's *t*-test was used to compare the difference between the two groups. The comparison between multiple groups was done using One-way ANOVA test followed by Post-Hoc Test (Least Significant Difference). The Chi-squared test was used to assess the relationship between miR-19 expression and clinicopathological features. *p*-values of <0.05 were considered to have statistical significances.

Results

MiR-19 Higher Expression in PDAC and Related to the Poor Prognosis of Patients

For understanding miR-19's role in PDAC progression, we firstly examined the miR-19 expression in PDAC tissues. As Figure 1A showed that miR-19 expression was observably higher in PDAC tissues. Moreover, we also found that miR-

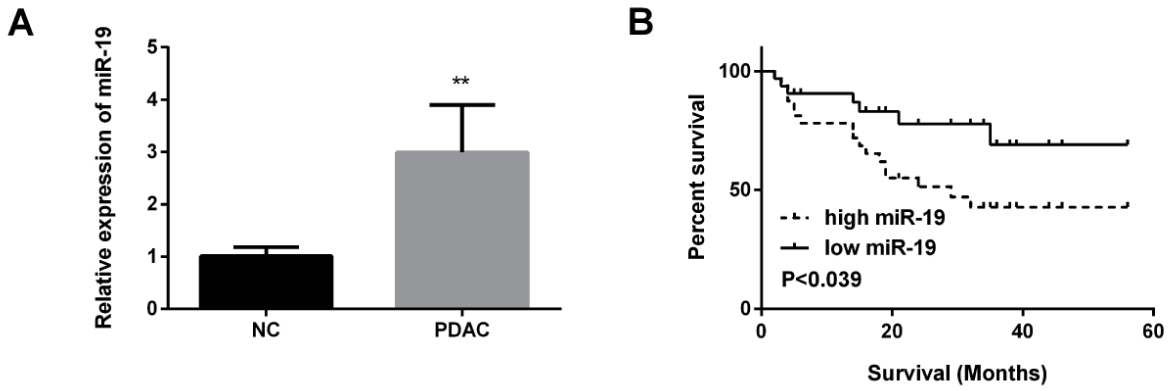

Figure 1. MiR-19 expression measured in PDAC tissues. **A,** High expression of miR-19 in PDAC tissues examined by qRT-PCR. **B,** MiR-19 expression and overall survival (OS) in BC patients were negatively correlated. ***p*<0.01.

19 was associated with tumor size, clinical stage, and lymph node metastasis as shown in Table I. However, the Kaplan-Meier survival curve stated that the higher the miR-19 expression, the poorer prognosis of PDAC patients, whereas the lower the miR-19 expression, the good prognosis of PDAC patients (p=0.0339, Figure 1B). Thus, we concluded that miR-19 might predict the prognosis of PDAC patient.

The Related Connection Between miR-19 Expression and Clinicopathological Features of PDAC Patients

In addition to detecting miR-19 expression, the relation between miR-19 and the clinicopathological characteristics of PDAC patients was investigated. Based on the average expression level of miR-19, miR-19 expression was divided into high and low expression group. According to the Chi-squared test, we also showed that miR-19 expression was correlated with TNM stage (p=0.003), tumor size (p=0.0169) and lymph node metastasis (p=0.007). However, there was no significant correlation between miR-19 expression and other clinical parameters, such as age, gender, and so on, as shown in Table I.

MiR-19's Promotion Effect on PDAC Cell Progression

To detect the miR-19 functional role in PDAC progression, we first examined miR-19 expression in PDAC cell lines; the results showed that miR-19 expression was increased significantly in both tumor cell lines in comparison with control cells (Figure 2A). Then, miR-19 mimic, inhibitor or the corresponding negatively control was transfected into PANC-1 cells, which increased or inhibited miR-19 expression. MiR-19 expression in PANC-1 cells was detected by qRT-PCR and was demonstrated to be significantly increased in the cells transfected with miR-19 mimic but decreased with miR-19 inhibitor compared with the control (Figure 2B). The cell proliferation in PANC-1 cells was tested by MTT assay and the cells were demonstrated to be significantly increased after they were transfected with miR-19 mimic but decreased with miR-19 inhibitor (Figure 2C). The transwell analysis was applied for assessing the migrated and invaded cells. As shown in Figure 2D-2E, the re-expression of miR-19 enhanced the cell migratory and invasive ability but the silence of miR-19 decreased the cell migratory and invasive ability of PDAC cells.

MiR-19's Promotion Effect on Tumor Growth In Vivo

To explore miR-19's effect on tumor growth, the PANC-1 cells with miR-19 plasmid or miR-NC was injected into the nude mice subcutaneously. Subsequently, we measured tumor volume every four days. Results showed that re-expression of miR-19 significantly increased the tumor volume contrast to the control group (Figure 3A). Moreover, the tumors with miR-19 plasmid grew more fastly than that with miR-NC (Figure 3B). These findings showed that miR-19 facilitated PDAC tumor growth *in vivo*.

The Direct Target of MiR-19 in PDAC Cells Was PTEN

To confirm the hypothetical target of miR-19, TargetScanHuman7.1 was firstly used (Figure 4A). Luciferase reporter assay was then used to verify this prediction in PDAC cells. The luciferase intensity in miR-19 mimic group was significantly reduced compared to control group in wild-type as expected, while there was no significant difference in mut-type (Figure 4B). Subsequently, PTEN expressional level in PDAC cells after re-expression or knockdown of miR-19 examined by RT-PCR was shown in Figure 4C. RT-PCR analysis showed that PTEN expression was markedly decreased in miR-19 mimic group, and miR-19 inhibitor up-regulate PTEN expression significantly (Figure 4D). Finally, we detected the relationship between miR-19 and PTEN and found that the fold change of miR-19 mRNA and PTEN was negatively correlated (Figure 4E).

Lower Expression of PTEN in PDAC and Related to the Good Prognosis of Patients

For understanding PTEN's role in PDAC progression, we firstly examined the PTEN expression in PDAC tissues using by IHC assays. As Figure 1A showed, the positive PTEN protein was detected in the nucleus of PDAC tissues (Figure 5A). Moreover, the protein intensity of PTEN was significantly decreased in PDAC tissues compared with the adjacent normal tissues (Figure 5B). However, Kaplan-Meier survival curve stated that the higher the PTEN expression, the good prognosis of PDAC patients, whereas the lower the PTEN expression, the poorer prognosis of PDAC patients (p=0.0147, Figure 5C). Thus, we concluded that PTEN might predict the prognosis of PDAC patient.

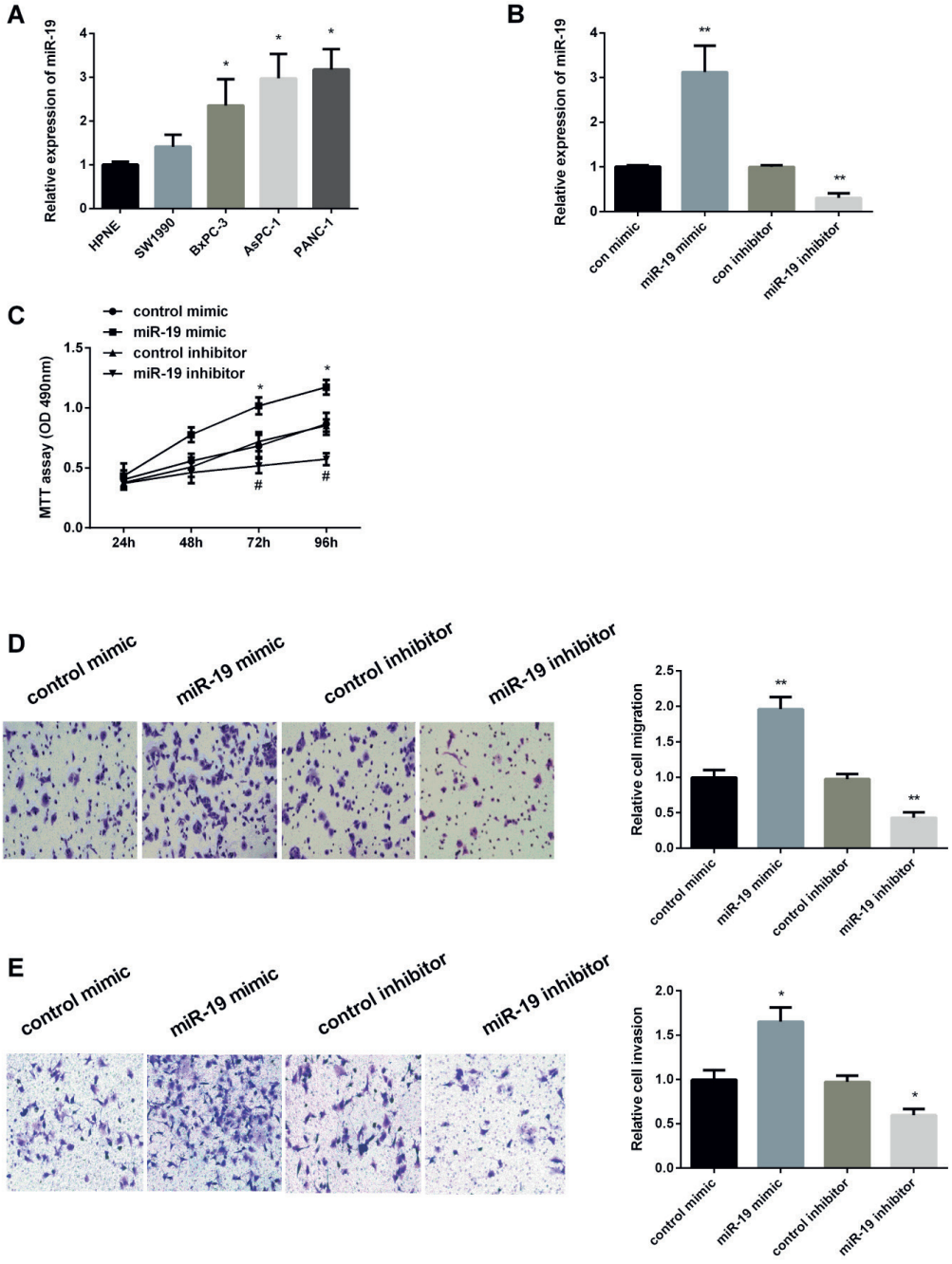

Figure 2. The role of miR-19 examined in PDAC cell proliferation, migration, and invasion. **A,** MiR-19 high expression tested in PDAC cell lines. **B,** MiR-19 expression in PANC-1 cells detected by qRT-PCR after treated with miR-19 mimic or inhibitor. **C,** Increased or decreased PDAC cells viability after treating with miR-19 mimic or inhibitor by MTT assay. **D-E,** The increased or decreased cell migration and invasion rate after treating with miR-19 mimic or inhibitor in PANC-1 cells by transwell assay (40×). ** $p<0.01$.

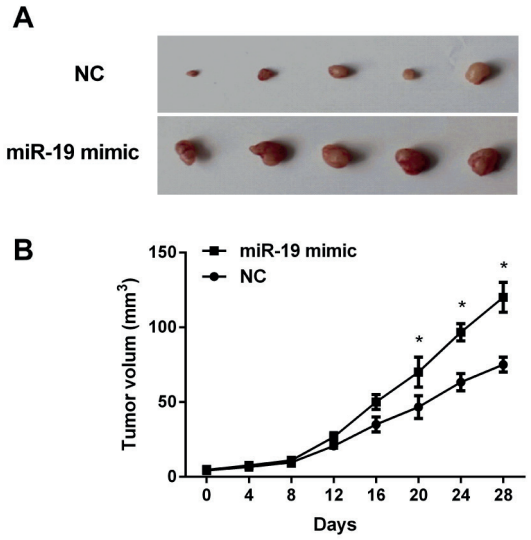

Figure 3. The effect of miR-19 on the tumor growth *in vivo*. **A,** The increased tumorigenic ability treated with miR-19 plasmid. **B,** The highly growth rate of tumors treated with miR-19 plasmid compared with control. ** $p<0.01$.

MiR-19 Regulated PI3K/AKT Signaling Pathway in PDAC Cells

Finally, the precise molecular mechanism of miR-19 in PDAC was studied. Firstly, we detected miR-19 effects on EMT and PI3K/AKT signaling pathway, which is considered to be an important pathway in PDAC development. Western blot results showed that the N-cadherin and Vimentin expression was facilitated while E-cadherin expression was declined by miR-19 mimic (Figure 6A). Inversely, the N-cadherin and Vimentin expression was decreased while E-cadherin expression was increased by a miR-19 inhibitor (Figure 6B). Thus, miR-19 regulated the cell invasion and migration by regulating EMT in PDAC. Secondly, we detected AKT and p-AKT protein expression in PANC-1 cells after treated with miR-19 mimic or inhibitor to further explore the underlying mechanism of miR-19 on cell proliferation. Western blot results showed that the phosphorylation of AKT expression was increased remarkably by miR-19 mimic (Figure 6C). On the contrary,

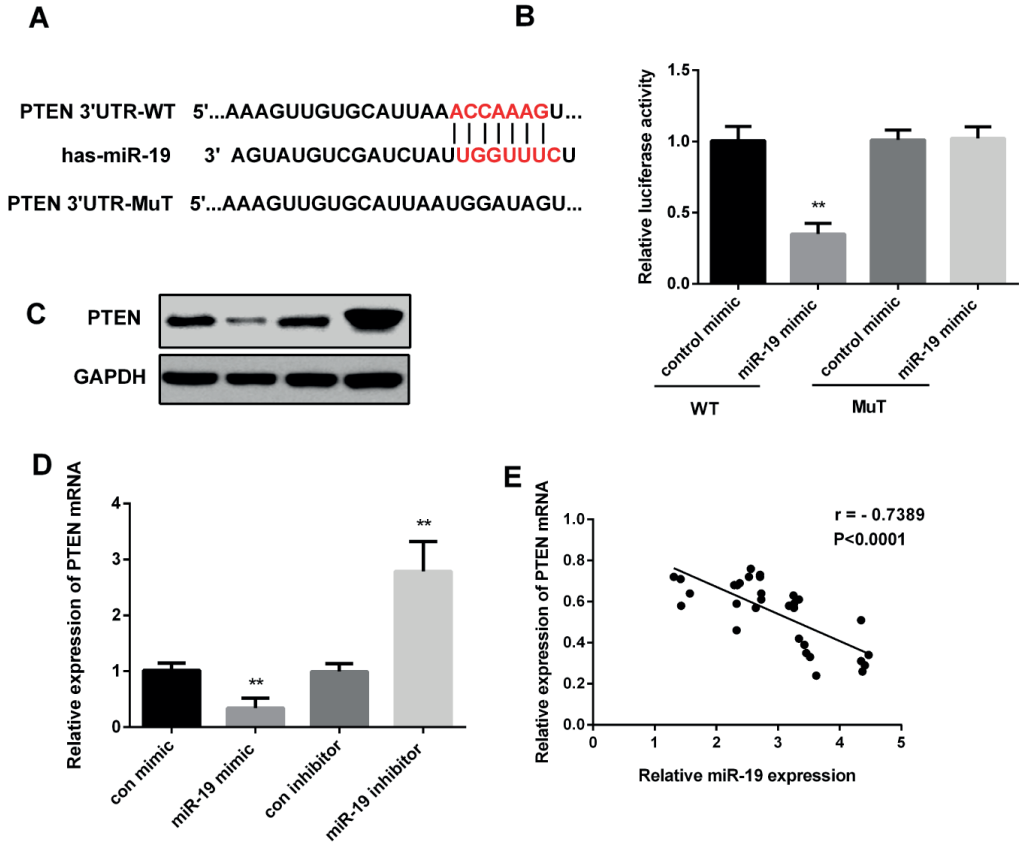

Figure 4. MiR-19's target detected in PDAC cells. **A,** Prediction of the binding site of miR-19 with PTEN. **B,** Down-regulated of luciferase activities in PANC-1 cells after transfected with PTEN-3'-UTR-wild. **C,** Down-regulated or up-regulated of PTEN mRNA expression in PANC-1 cells after treated with miR-19 mimic or inhibitor. **D,** The negatively correlation between PTEN and miR-19 expression in PDAC tissues (r=-0.7389, $p<0.0001$) ** $p<0.01$

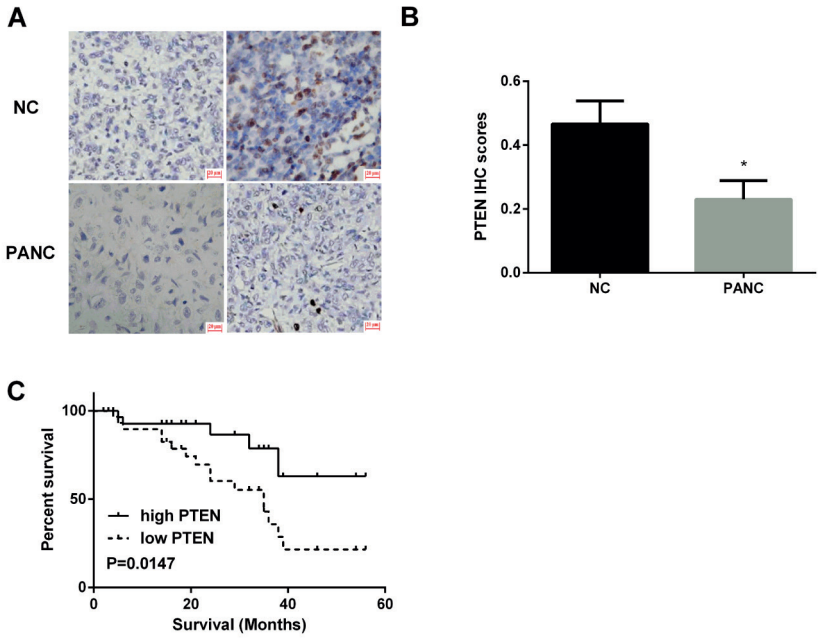

Figure 5. The expression of PTEN in PDAC tissues. **A-B,** Decreased of PTEN protein expression detected in PDAC tissues by immunohistochemistry **C,** PTEN expression and overall survival (OS) in PDAC patients were positive correlated (400×). ***p*<0.01.

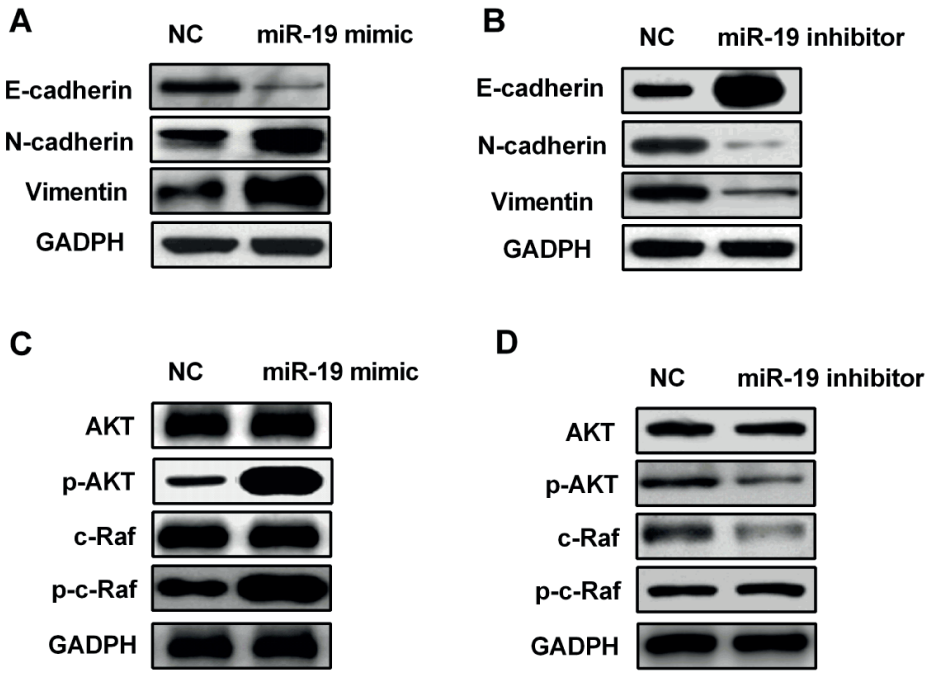

Figure 6. MiR-19 regulated EMT and AKT pathway in PDAC cell progression. **A-B,** Western blot analysis of low expression E-cadherin, N-cadherin and high expression of Vimentin in PANA-1 cells after treated with miR-19 mimic or inhibitor. **C-D,** Western blot analysis of AKT and the phosphorylation of p-AKT in PANC-1 cells after treated with miR-19 mimic or inhibitor.

the phosphorylation of AKT expression was decreased remarkably by a miR-19 inhibitor (Figure 6D). Taken together, miR-19 was examined to regulate EMT and AKT pathway in PDAC progression.

Discussion

PDAC is one of the worst prognosis of malignant tumors, with mortality and morbidity roughly the same in the United States[1] and China[20]. There is growing evidence that the EMT process plays an important role in the invasion and metastasis of various types of tumors, including PDAC. However, the mechanisms underlying this malignant transformation have not been fully demonstrated. The new report on miRNA behavior reveals its essential role in carcinogenesis and development. This study provides clinical and experimental evidence for the role of miR-19 in PDAC. We found a high expression of miR-19 in PDAC cells, which is associated with the overall survival, tumor size, TNM stage, and lymph node metastasis. The results were similar to the previous research that miR-19 was up-regulated in PDAC cells[13,21]. Moreover, miR-19 was proved to promote cell migration and invasion. The promoter effect of miR-19 was also found in colorectal cancer progression[13] and osteosarcoma (OS) development.

PTEN was known as a tumor suppressor and took part in a variety of tumors[18,22,23]. It was also proved to act as a target of miRNAs in modulating cancers progression. For example, PTEN was the target of miR-28 and suppressed gastric cancer cell progression[24]. Also, it inhibited renal cancer cell viability as a target of miR-30a[25]. PTEN was reported by Zhao et al[26] to regulate OS cell growth and apoptosis as a target of miR-19. Moreover, PTEN down-regulated PDAC cells metastasis as a target of miR-107[27]. In our study, we first demonstrated that PTEN inhibited PDAC cell progression and acted as a direct target of miR-19. We also demonstrated that miR-19 promoted the protein level associated with EMT and PI3K/AKT pathway.

Conclusions

For the first time, we demonstrated that miR-19 targeted PTEN to enhance PDAC cell progression *via* PI3K/AKT signaling. Therefore, miR-19 represented a novel therapeutically relevant cellular target for the treatment of PDAC patients.

Conflict of Interests

The Authors declare that they have no conflict of interests.

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