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MiR-497 inhibits cell proliferation and invasion ability by targeting HMGA2 in pancreatic ductal adenocarcinoma

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Abstract. – OBJECTIVE: MicroRNAs have been implicated to play a crucial regulating role in human cancers. The study aims to explore the role and clinical significance of miR-497 in pancreatic ductal adenocarcinoma (PDAC).

PATIENTS AND METHODS: The relative expression of miR-497 in human PDAC tissue samples and adjacent normal tissues was measured using the quantitative Real Time-Polymerase Chain Reaction (qRT-PCR). Cell Counting Kit (CCK-8) assay, cell migration, and invasion assays were performed to detect cell proliferation and invasion ability. Downstream target gene was confirmed by using luciferase activity assays. QRT-PCR and Western blotting assays also were performed.

RESULTS: We found that miR-497 expression was significantly downregulated in PDAC tissues and cells. Lower miR-497 expression associated with lymph node metastasis and predicts a poor prognosis in PDAC patients. In *in vitro* assay, we demonstrated that miR-497 overexpression inhibited cell proliferation, migration, and invasion of PDAC. Furthermore, we demonstrated that HMGA2 was a direct target of miR-497 in PDAC cells. MiR-497 inhibited cell proliferation and invasion by regulating HMGA2 expression.

CONCLUSIONS: Our results indicated that miR-497 may serve as a predictor for PDAC and could be a novel target of PDAC treatment.

Key Words:

MicroRNAs, PDAC, MiR-497, HMGA2, Cell invasion.

Introduction

Pancreatic cancer is a leading cause of cancer-related deaths worldwide and shows poor prognosis in patients[1]. Despite large developments in the detection and management of pancreatic cancer, the five-year overall survival rate has slightly improved[2]. Therefore, to further investigate the underlying molecular mechanisms is urgently needed.

MicroRNAs (miRNAs) are small non-coding RNAs and inhibit their target gene expression by regulating the 3'untranslated region (3'-UTR) to induce messenger RNA (mRNA) silence or translational inhibition[3,4]. Recent studies show that miR-497 plays an important role in various cancers. Such as, miR-497 suppresses epithelial-mesenchymal transition and metastasis in colorectal cancer cells by targeting fos-related antigen-1[5]. MiR-497 inhibited proliferation, migration, and invasion of thyroid papillary carcinoma cells by negatively regulating YAP1 expression[6]. MicroRNA-497 inhibits tumor growth by targeting insulin receptor substrate 1 in colorectal cancer[7]. However, the role and clinical significance of miR-497 expression in pancreatic ductal adenocarcinoma (PDAC) remain unknown.

Here, we found that miR-497 was significantly downregulated in PDAC tissues and cells. *In vitro*, we also demonstrated that miR-497 overexpression inhibited cell proliferation, migration, and invasion of PDAC. Moreover, we showed that HMGA2 was a direct target of miR-497 in PDAC cells. MiR-497 inhibited cell proliferation and cell invasion by regulating HMGA2 expression in PDAC. Thus, miR-497 may be a novel target of PDAC treatment.

Patients and Methods

Patient Tissues and Ethics Statement

A total of 42 fresh tumor and matched adjacent normal tissue samples were obtained from patients pathologically confirmed as PDAC at the Second Hospital of Hebei Medical University between January 2012 and June 2017 who were treated with neither radiation nor chemotherapy before resection. Written informed consents were obtained from the patients before surgery. This investigation was approved by the Ethics Committee of the Second Hospital of Hebei Medical University.

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Cell Lines Culture

The three human PDAC cell lines Panc1, AsPC1, and Panc28 were obtained from the Cell Bank, Type Culture Collection of the Chinese Academy of Sciences (Shanghai, China). The human immortal ductal cell line HPDE was obtained from American Type Culture Collection (Manassas, VA, USA). Dulbecco's Modified Eagle's Medium (DMEM; Gibco, Grand Island, NY, USA) with 10% fetal bovine serum (FBS; Gibco, Grand Island, NY, USA) was used to culture cell lines in a humidified 5% CO_2 atmosphere at 37°C.

RNA Extraction and Quantitative Reverse Transcription PCR (QRT-PCR)

TRIzol™ reagent (Invitrogen, Carlsbad, CA, USA) was used for RNA extraction from cell and tissues according to the manufacturer's instructions. QRT-PCR analysis was performed using a Prime Script RT reagent kit (TaKaRa Biotechnology Co., Ltd., Dalian, China) and SYBR® Premix Ex Taq™ II (TaKaRa Biotechnology Co., Ltd., Dalian, China). Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was used as an endogenous control. QRT-PCR was performed on a CFX96 Real-Time PCR Detection System supplied with analytical software (Bio-Rad Laboratories, Hercules, CA, USA). PCR conditions were as follows: 40 cycles of 95°C for 15 s, 60°C for 15 s, and 72°C for 45 s. The primers were as follows: miR-497-forward (F), 5'-ACACTCCAGCTGGGCAGCAGCACACTGTGG-3'; miR-497-reverse (R), 5'-TGGTGTCGTGGAGTCG-3'; U6-forward (F), 5'-AACGCTTCACGAATTTGCGT-3';U6-F,5'-CTCGCTTCGGCAGCACA-3'; and U6-reverse (R), 5'-TGGTGTCGTGGAGTCG-3'.

Transwell Migration and Invasion Assays

The invasion assay was performed using Matrigel™ Invasion Chambers (Millipore, Billerica, MA, USA) according to the manufacturer's instructions. After cell transfection at 24 h, 50,000 cells per well with serum-free DMEM were seeded in the upper chambers, and DMEM with 10% FBS was added to the lower chambers. After 48 h, the cells were fixed with 4% paraformaldehyde and then stained with crystal violet.

Western Blot Analysis

Proteins were extracted using radioimmunoprecipitation lysis buffer (RIPA; Invitrogen, Carlsbad, CA, USA) and the protein was determined using the Bicinchoninic Acid Protein assay kit (BCA; Beyotime Institute of Biotechnology, Nantong, Jiangsu, China). Equal amounts of the protein (30 µg) were subjected to 10% gels sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) and then transferred to polyvinylidene difluoride membranes (PVDF; Millipore Billerica, MA, USA). The membranes were blocked with 5% non-fat milk for 1 h at room temperature, followed by incubation with primary antibodies against HMGA2 (1:500; Abcam, Cambridge, MA, USA) and GAPDH (1:2000; Beyotime Institute of Biotechnology, Nantong, Jiangsu, China) overnight at 4°C. Then, the membranes were incubated with horseradish peroxidase (HRP)-conjugated goat anti-rabbit secondary antibodies (1:2000; Santa Cruz Biotechnology, Santa Cruz, CA, USA) at room temperature for 2 h. The proteins of interest were detected by the enhanced chemiluminescence (ECL) detection system and analyzed by using Image-Pro Plus 6.0 software (Media Cybernetics; Rockville, MD, USA). GAPDH was served as the loading control.

Luciferase Reporter Assay

Prediction of miR-497 binding sites was performed using miRanda (www.microrna.org) to predict biological targets of miRNAs by searching for the presence of conserved 8mer, 7mer, and 6mer sites that match the seed region of each miRNA. A fragment of 3'-UTR of HMGA2-WT or HMGA2-MUT containing the putative miR-497 binding site was constructed and inserted into pMIR-REPORTER (Promega, Madison, WI, USA) and validated by DNA sequencing. Constructs were transfected into Panc28 cells in 24-well plates and co-transfected with miR-497 mimic or miR-NC. Luciferase assays were performed 24 h post-transfection using the Dual-Luciferase Reporter Assay system (Promega, Madison, WI, USA).

Statistical Analysis

Data was performed using SPSS version 17.0 statistical software package (SPSS Inc., Chicago, IL, USA). Data are shown as the mean ± standard deviation (SD). Kaplan-Meier analysis with a log-rank test was applied for survival analysis. Chi-square test was used for categorical variables. Clinicopathological parameters were evaluated using the Chi-squared test. Student's t-tests were used to compare group means. A $p < 0.05$ was considered statistically significant.

Results

MiR-497 Expression Is Downregulated in Pancreatic Ductal Adenocarcinoma (PDAC)

We detected the miR-497 expression using qRT-PCR analysis in 42 cases of PDAC tissues and adjacent normal tissues. As shown in Figure 1A, miR-497 expression was downregulated in PDAC tissue samples compared to that in adjacent normal tissue samples ($p<0.05$). Furthermore, we detected the expression of miR-497 among Panc1, AsPC1, and Panc28 cell lines and the human immortal ductal cell line HPDE. The expression of miR-497 is significantly decreased in several tumor cell lines compared to that in HPDE cells ($p<0.05$; Figure 1B). Based on the median expression of miR-497 in PDAC tissues, we divided the patients into higher miR-497 expression groups and lower miR-497 expression groups. Chi-square test was used for the analysis among clinicopathological data. The results showed that miR-497 expression was significantly associated with lymph node metastasis ($p=0.011$, Table I). Kaplan-Meier analysis with a log-rank test showed lower miR-497 expression predicts a poor prognosis in PDAC patients (Figure 1C, $p<0.05$). Thus, these results demonstrated that miR-497 expression was lower in human PDAC.

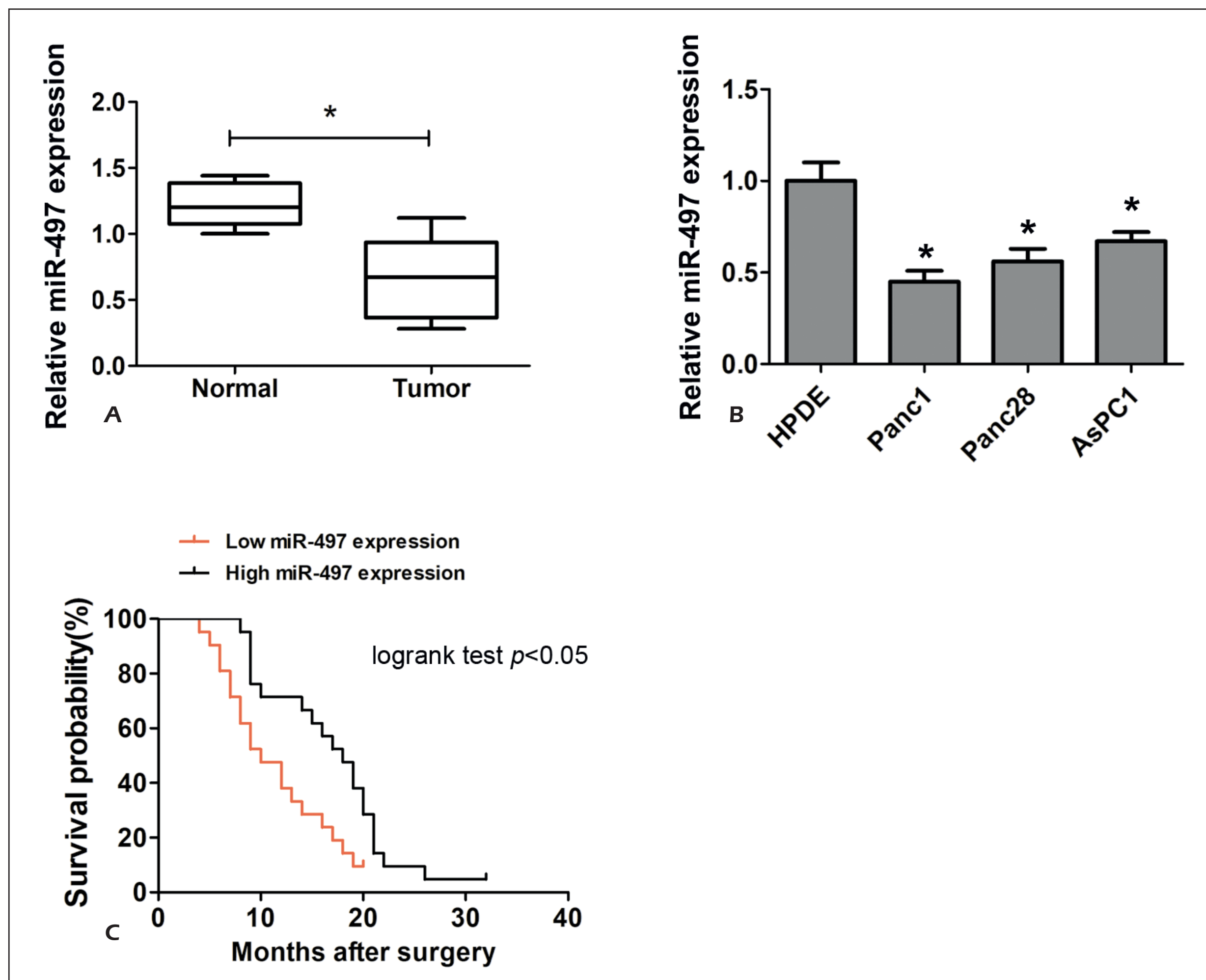

Figure 1. MiR-497 was lower expression in PDAC and associated with poor prognosis in patients. **A**, MiR-497 expression in PDAC tissues and adjacent normal tissues was detected by qRT-PCR. Statistical differences between PDAC tissues and paired adjacent normal tissues were analyzed using paired samples t-test. **B**, MiR-497 expression was detected in three human PDAC cell lines Panc1, AsPC1, and Panc28 cell and a human immortal ductal cell line HPDE. **C**, Lower miR-497 expression predicted a poor prognosis in PDAC compared to higher miR-497 expression in PDAC patients, log-rank test *$p<0.05$.

Table I. Clinicopathological features and the expression of PGM5-AS1 in ccRCC patients.

	MiR-497 expression levels			
Characteristics	**Patients (n=42)**	**Low (n=21)**	**High (n=21)**	***p*-value**
Sex				0.204
Male	26	15	11	
Female	16	6	10	
Age (years)				0.747
≤ 55	15	7	8	
> 55	27	14	13	
Tumor size				0.352
≤ 4 cm	19	11	8	
> 4 cm	23	10	13	
Differentiation				0.495
Well and moderately	30	14	16	
poor	12	7	5	
Lymph node metastasis				0.011 a
No	25	8	17	
Yes	17	13	4	
Pathologic T category				0.525
T1, T2	16	7	9	
T3, T4	26	14	12	

[a]*p*-value <0.05.

MiR-497 Inhibits Cell Proliferation, Migration, and Invasion Ability In Vitro

To further demonstrate the functional role of miR-497 in PDAC cells, miR-497 was upregulated or downregulated by miR-497 mimic or miR-497 inhibitor in Panc1 and Panc28 cells (Figure 2A-2B). Then, we detected cell proliferation, migration, and invasion by CCK-8 and transwell assays. The CCK-8 assays results showed that miR-497 mimic inhibits cell proliferation compared to control groups in Panc1 and Panc28 cells, while miR-497 inhibitor promotes cell proliferation compared to control groups (Figure 2C-2D). The results of transwell migration ability assays results indicated that miR-497 mimic inhibits cell migration compared to control groups in Panc1 and Panc28 cells, while miR-497 inhibitor promotes cell migration ability compared to control groups (Figure 3A-3B). Similarly, the results of transwell invasion showed that miR-497 mimic inhibits cell invasion ability, but miR-497 inhibitor promotes cell invasion ability (Figure 3C-3D). These results indicated that miR-497 could inhibit cell proliferation, migration, and invasion in PDAC.

HMGA2 Is a Directs Target of MiR-497 in PDAC Cells

It is well established that miRNAs could play important roles in tumor progression by inhibiting their target genes. Through miRanda database (www.microRNA.org) analysis, the results showed the significant sequence complementarity between miR-497 and HMGA2 (Figure 4A). We constructed the wild type HMGA2 (HMGA2-WT) and mutant type HMGA2 (HMGA2-MUT) luciferase reporter vectors. The luciferase reporter assay results showed that miR-497 mimic significantly decrease HMGA2-WT activity, while it had no effect on HMGA2-MUT (Figure 4B). Furthermore, we demonstrated that miR-497 mimic in Panc1 and Panc28 significantly decreased HMGA2 expression by mRNA and protein levels (Figure 4C-4F). Thus, these results indicated that HMGA2 is a target of miR-497 in PDAC cells.

MiR-497 Inhibits Cell Proliferation and Invasion Ability by Regulating HMGA2

We demonstrated that HMGA2 expression was upregulated in PDAC tissues compared to adjacent normal tissues (Figure 5A). HMGA2 expression was downregulated by transfecting siRNA-HMGA2 in Panc1 cells (Figure 5B). Our results showed that downregulated HMGA2 expression could inhibit cell proliferation and invasion in Panc1 cells, but was dismissed by co-transfecting with miR-497 inhibitor (Figure 5C-5D). Thus, these results showed that miR-497 inhibits cell proliferation and invasion ability by regulating HMGA2.

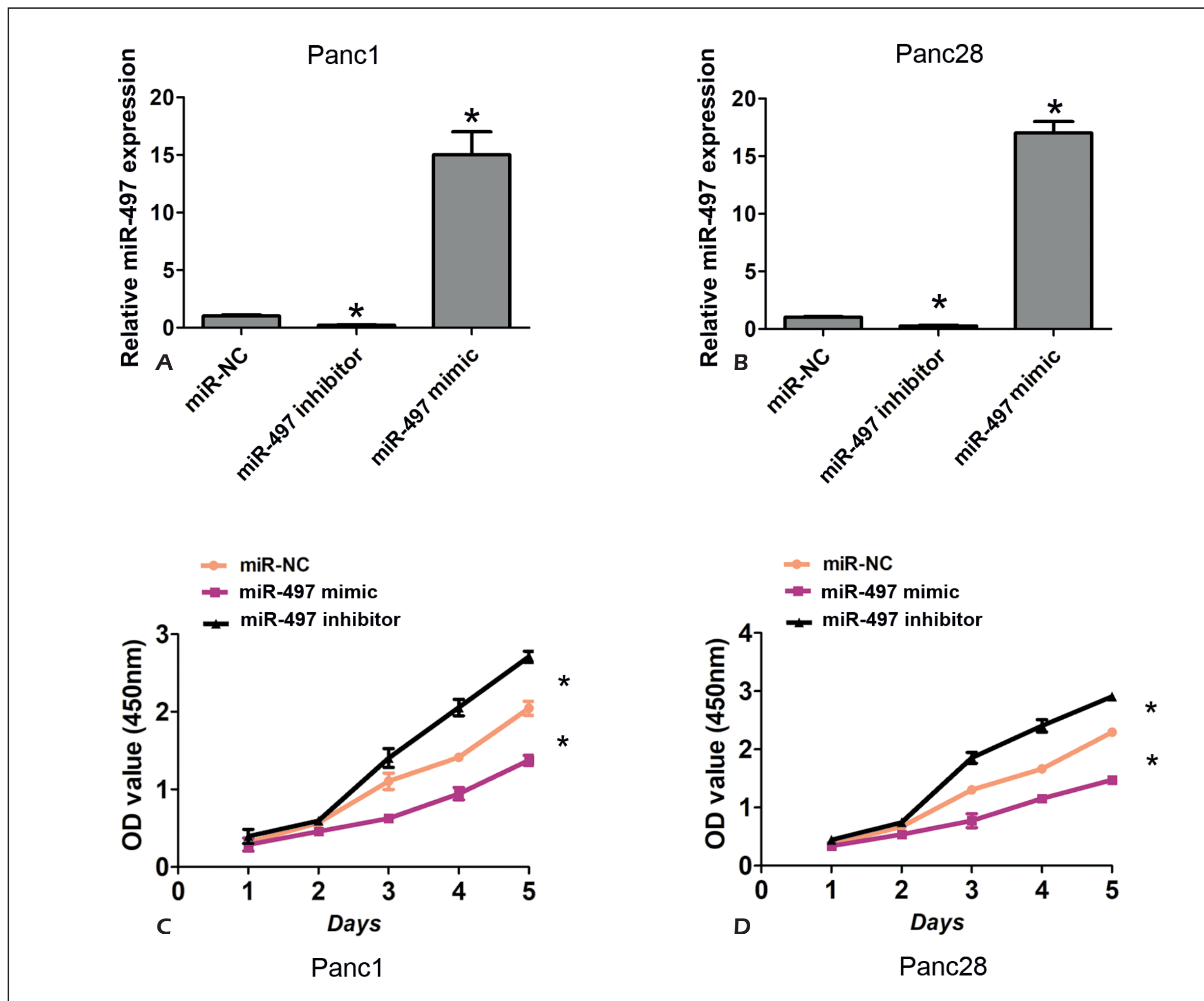

Figure 2. MiR-497 inhibited cell proliferation ability in PDAC cells. **A-B**, MiR-497 expression was detected when cells were transfected with miR-NC, miR-497 mimic or miR-497 inhibitor in Panc1 and Panc28 cells. **C-D**, CCK-8 assay was performed after Panc1 and Panc28 cells were transfected with miR-NC, miR-497 mimic or miR-497 inhibitor at 1,12, 24, 48, and 72 h. Error bars indicate the mean ± SD. $^*p<0.05$.

Discussion

MiRNAs act as post-transcriptional gene regulators in tumor progression, including cell apoptosis, cell proliferation, cell migration, and cell invasion[8]. In recent years, the indisputable oncogenic roles of miR-497 have played important roles in tumors[9]. MicroRNA-497 inhibits tumor growth by targeting insulin receptor substrate 1 in colorectal cancer[7]. MicroRNA-497 accelerates apoptosis while inhibiting proliferation, migration, and invasion through negative regulation of the MAPK/ERK signaling pathway via RAF-1[10]. MiR-497 suppresses epithelial-mesenchymal transition and metastasis in colorectal cancer cells by targeting fos-related antigen-1[5]. Overexpression of microRNA-497 suppresses cell proliferation and induces apoptosis by targeting paired box 2 in human ovarian cancer[11]. MicroRNA-497 inhibits the proliferation, migration, and invasion of human bladder transitional cell carcinoma cells by targeting E2F3[12]. Reciprocal interplay of miR-497 and MALAT1 promotes tumorigenesis of adrenocortical cancer[13]. MicroRNA-497 increases apoptosis in MYCN amplified neuroblastoma cells by targeting the key cell cycle regulator WEE1[14]. However, the role of miR-497 expression in PDAC remains unknown.

Here, we found that miR-497 was significantly downregulated in PDAC tissues and cells. A lower miR-497 expression associated with lymph node metastasis and predicts a poor prognosis in PDAC patients. In *in vitro* assay, we demonstrated

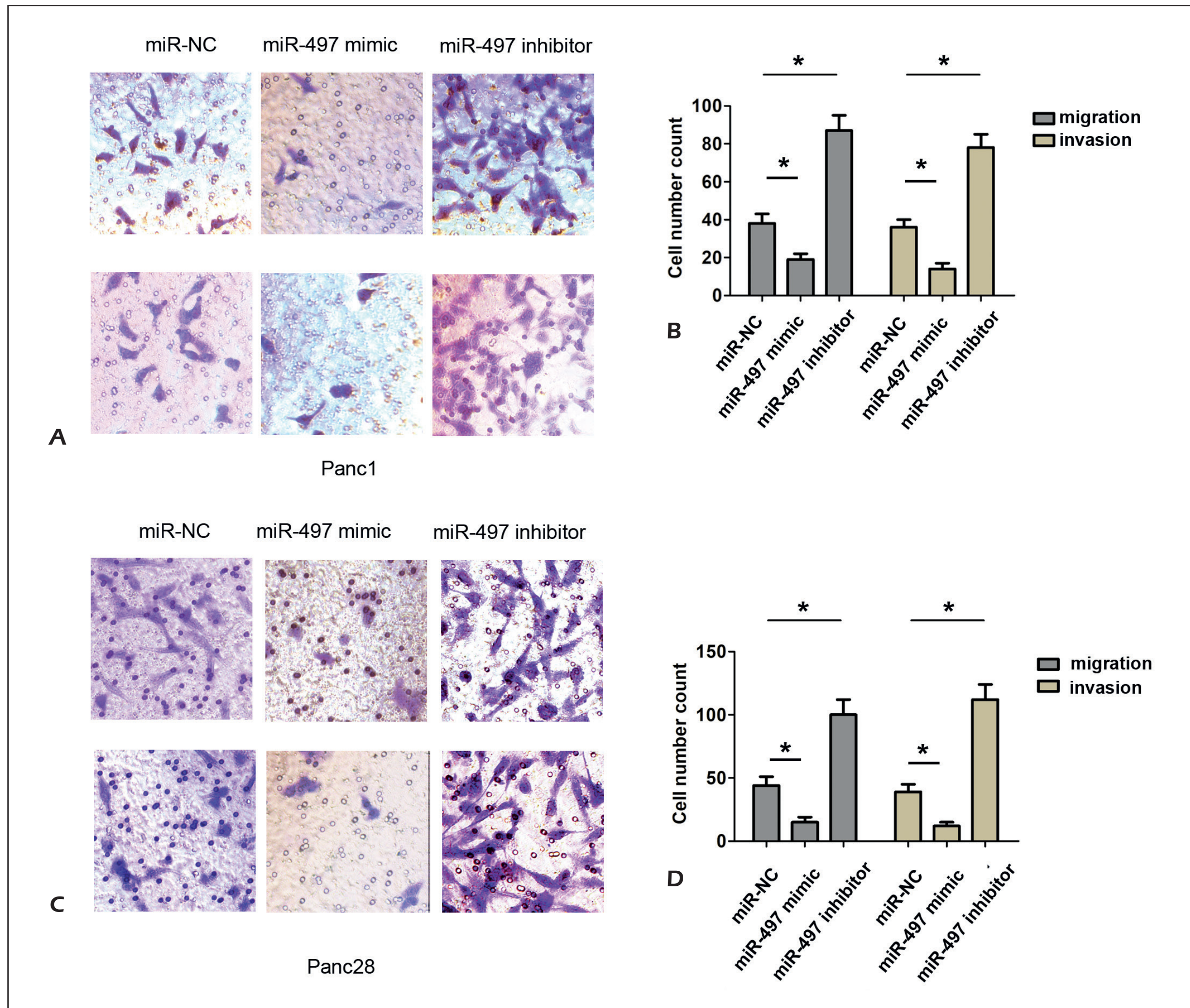

Figure 3. MiR-497 inhibited cell migration and invasion ability in PDAC cells. **A-B**, Transwell migration and invasion assays were performed after Panc1 cells were transfected with miR-NC, miR-497 mimic or miR-497 inhibitor (magnification 200 X), *$p<0.05$. **C-D**, Transwell migration and invasion assays were performed after Panc28 cells were transfected with miR-NC, miR-497 mimic or miR-497 inhibitor at 48 h. Error bars indicate the mean ± SD. Magnification 200 X, *$p<0.05$.

that miR-497 overexpression inhibited cell proliferation, migration, and invasion of PDAC.

HMGA2 has been found to act as an oncogene in pancreatic cancer. Such as, HMGA2 expression correlates with advanced tumor grades, lymph node metastasis, and poor prognosis and regulating epithelial-mesenchymal transition in pancreatic cancer[15]. CXCR4/Let-7a axis regulates metastasis and chemoresistance of pancreatic cancer cells through targeting HMGA2[16]. High mobility group A2 (HMGA2), a non-histone protein, can promote epithelial-mesenchymal transition (EMT) via MAPK pathway in prostate cancer[17]. Overexpression of HMGA2 enhanced TGFβ signaling by activating expression of the TGFβ type II receptor, which also localized to the invasive front of tumors in breast cancer[18]. In the study, the luciferase reporter assay results showed that miR-497 mimic significantly decrease HMGA2-WT activity, while it had no effect on HMGA2-MUT. Additionally, we demonstrated that HMGA2 expression was upregulated in PDAC tissues compared to adjacent normal tissues. Furthermore, we demonstrated that miR-497 mimic in Panc1 significantly decreased HMGA2 expression. Downregulated HMGA2 expression could inhibit cell proliferation and invasion in Panc1 cells-but was dismissed by co-transfecting with miR-497 inhibitor. Thus, these results showed that miR-497 inhibits cell proliferation and invasion ability by regulating HMGA2. Thus, these results indicated that HMGA2 is a target of miR-497 in PDAC cells.

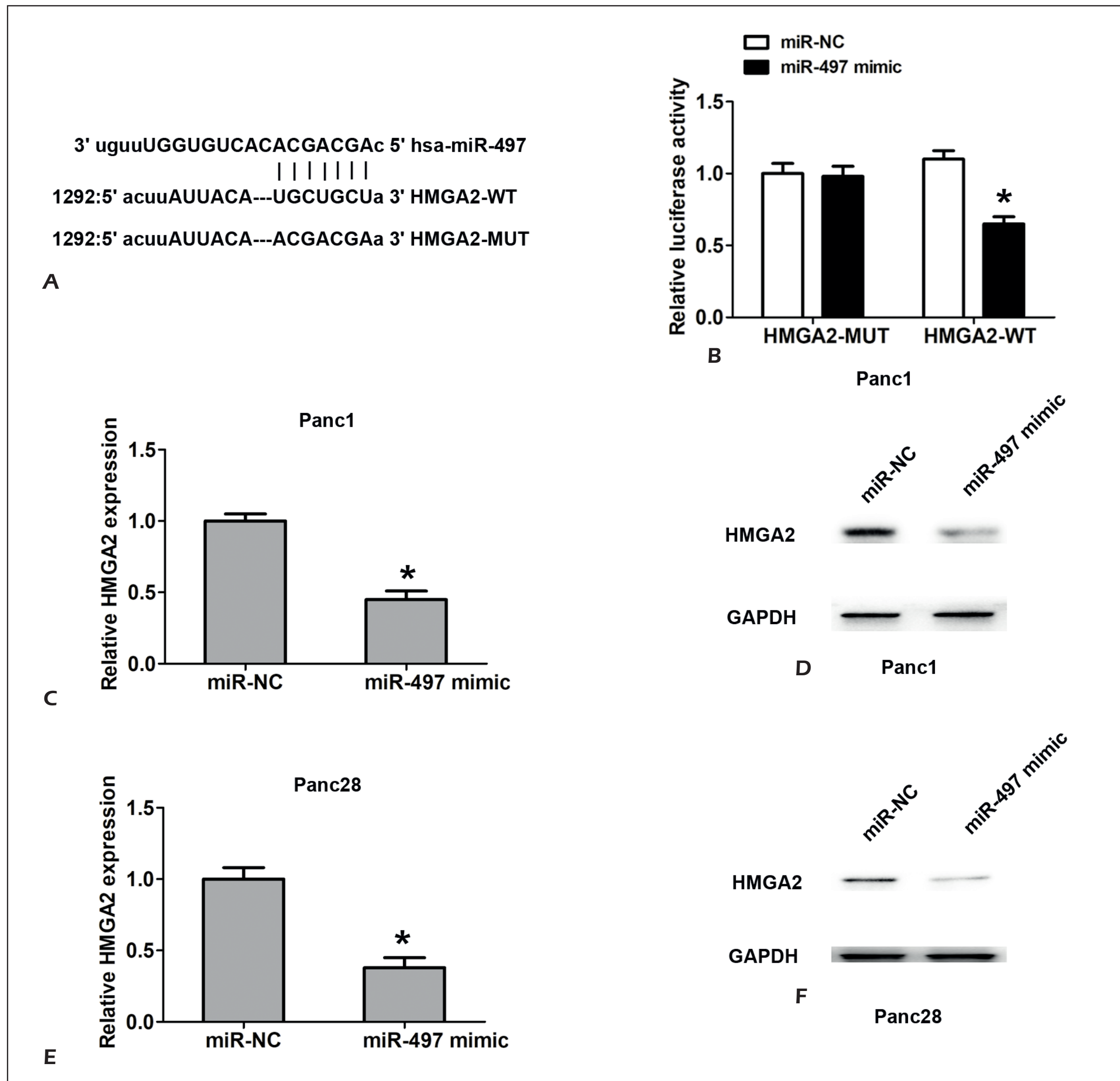

Figure 4. HMGA2 is a target of miR-497 in PDAC cells. **A**, Wild type 3'-UTR of HMGA2 mRNA containing binding sites with miR-497 and mutant sites were cloned into pMIR-REPORTER Dual-Luciferase vector. **B**, Panc1 cells were co-transfected with wild type 3'-UTR of HMGA2 or mutant type 3'-UTR of HMGA2 and miR-497 mimic or miR-NC. At 48 hours after transfection, the activity of firefly luciferase was measured by using the Dual-Luciferase reporter assay system. **C-D**, Relative mRNA and protein expression of HMGA2 was examined by qRT-PCR or Western blot analyses after transfection of miR-497 mimic in Panc1 cells, compared with control group. **E-F**, Relative mRNA and protein expression of HMGA2 were examined by qRT-PCR or Western blot analyses after transfection of miR-497 mimic in Panc28 cells, compared with control group. Error bars indicate the mean ± SD. *$p<0.05$.

Conclusions

MiR-497 was significantly downregulated in PDAC tissues and cells. In *in vitro* assay, we demonstrated that miR-497 overexpression inhibited cell proliferation, migration, and invasion of PDAC. Moreover, HMGA2 was the direct target of miR-497 in PDAC cells. MiR-497 inhibited cell invasion by regulating HMGA2 expression. Thus, miR-497 may be a novel target of PDAC treatment.

Conflict of Interests

The Authors declare that they have no conflict of interests.

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