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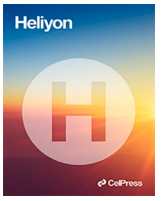

Research article

Long non-coding RNA MIR600HG as a ceRNA inhibits the pancreatic cancer progression through regulating the miR-1197/PITPNM3 axis

Baoming Yang, Zhikai Jiao, Ningning Feng, Yueshan Zhang, Shunxiang Wang *

Department of Hepatobiliary Surgery, The Fourth Hospital of Hebei Medical University, Shijiazhuang, 050000, China

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ABSTRACT

Objective: Pancreatic cancer (PC) is considered to be a highly malignant cancer with poor prognosis. Long non-coding RNAs (lncRNAs) is the potential factor to predict cancer prognosis. The effect of MIR600HG in PC needs to be further studied. Our work mainly focused on the importance of MIR600HG for PC prognosis and its underlying molecular mechanism of regulating PC progression.

Methods: Data set was acquired from TCGA database to find differentially expressed genes and prognostic significance of MIR600HG in PC, and to construct the MIR600HG competitive endogenous RNA (ceRNA). Clinical specimens were collected to prove the analysis results. Vector over-expressed MIR600HG was transfected to study the roles of MIR600HG in proliferation, apoptosis, invasion and migration. The methods of CCK-8, flow cytometry, Transwell and scratch assays were all used in order to explore the apoptosis, migration and invasion. We evaluated the proliferation-related genes (PCNA, CyclinD1 and P27), as well as invasion and migration-related genes such as MMP-9, MMP-7 and ICAM-1. The transcriptional regulation between MIR600HG and miR-1197/PITPNM3 axis was determined with luciferase reporter assays.

Results: In present study, MIR600HG was dropped in both PC tissues and cells, and the down-regulated MIR600HG was closely related to the poor clinical outcomes in PC patients. MIR600HG could inhibit proliferation, migration and invasion in PC cells. We also investigated whether MIR600HG acting as a sponge of microRNA-1197 (miR-1197) and miR-1197 acting on PITPNM3. We found the positive association between MIR600HG and PITPNM3, as well as the negative association of miR-1197 and MIR600HG (or PITPNM3). Moreover, PITPNM3 mRNA and protein expression saw a simultaneous increase after the MIR600HG-overexpression (MIR600HG-OE), but this result partially diminished in MIR600HG-OE cells and miR-1197 mimics.

Conclusions: Our study explored the anticancer action of MIR600HG in PC by regulating miR-1197 to increase the expression of PITPNM3, which might help the diagnosis and therapy of PC.

1. Introduction

Pancreatic cancer (PC) is one of the leading causes of cancer death worldwide. Despite the incidence of PC being much lower than that of other malignancies, such as lung, breast, colorectal, and prostate tumors, it was the third highest cause of cancer-related death

* Corresponding author.
E-mail address: gdwangsx@163.com (S. Wang).

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in 2021 and is possible to rise to the second position by 2030. As much as 52 % of pancreatic tumors show distant dissemination at diagnosis, with an overall life expectancy of 5 years at 3 %. Compared with other types of tumors, therapeutic progress in PC is scarce. Despite the success of immunotherapy and targeted therapies in many solid tumors in the last decade, these agents were not shown to provide a significant benefit to PC patients and chemotherapy is still the major therapeutic regimen [1,2]. The molecular heterogeneity of PC may demand as much diversified treatment approaches based on individual tumor characteristics. Thus, it is necessary to study the potential pathophysiology mechanisms and to develop more effective therapeutic strategies [3].

Long non-coding RNAs (lncRNAs) do not encode proteins, but can modulate gene expression and are involved in many disease development [4–7]. MIR600HG is a newly discovered lncRNA and rarely reported, which was found to promote the colorectal cancer development recently [8]. In addition, another study also showed that MIR600HG may be a biomarker for predicting the outcome of PC [9]. In our article, we found that MIR600HG could inhibit the proliferation, migration and invasion via the miR-1197/PITPNM3 axis in PC, which might be applied as a novel target for PC treatment.

2. Materials and methods

2.1. Genes data and differentially expressed gene analysis

The results of this article are based on the TCGA database to generate pancreatic cancer data sets. Genes expression data were got from the TCGA database using the R package "TCGAbiolinks". Genes with corrected p value less than 0.05 and expression change more than one-fold were considered to be differentially expressed

2.2. Clinical data and survival analysis

The clinical data set of PC patients was downloaded from TCGA database. The cancer samples were divided into two subsets according to the median levels. Kaplan-Meier (KM) survival curves were applied to analyze the survival difference. Log-rank test was applied to estimate statistical significance. Thereafter, we established the nomogram so as to predict the survival rate and the median survival time of patients at different time points. Tissue samples were acquired from the Fourth Affiliated Hospital of Hebei Medical University with the informed content of the enrolled patients. The ethics was approved by the Human Research Ethics Committee of Fourth Affiliated Hospital of Hebei Medical University.

2.3. Viability assay

The Cell Counting Kit-8 (CCK-8) assay (Solarbio, China) was carried out to evaluate the cell viability. Cells (1×10^5 cells per well) were plated in 96-well plates. The cell growth rate was analyzed by CCK-8. The optical density value was measured by a microplate reader at 570 nm.

2.4. Flow cytometry assay

The Cell cycle and apoptosis of transfected cells were carried out by flow cytometry assay. The cells were washed by phosphate buffer saline (PBS). Next, the pellet was resuspended, fixed in 70 % methanol, and stored overnight at 4 °C. We washed the cells again with PBS followed by addition of 200 μL staining solution (0.1 % [v/v] Triton X-100, 1 μg/mL DAPI in PBS). At last, the final mixture was incubated for 30 min in the dark environment.

2.5. Scratch assay

We used the 200-ml pipette tip to create a wound on the confluent cell monolayer. Then, the inverted microscope was applied to take images of wound closure at 24 h. Last, wound healing distance was study.

2.6. Migration and invasion assays

The transwell assays were conducted using a transwell chamber. The cells (1×10^5) were seeded on the upper compartment of the chamber and 800 μl DMEM with 10 % FBS were added to the lower chamber. Cells migrated from the upper chamber onto the lower chamber after 24 h transfection, Then, the cells were fixed with 4 % paraformaldehyde and stained with 0.1 % crystal violet. Finally, the migrated cells on the lower surface were photographed with a light microscope.

2.7. Quantitative real-time PCR

Total RNA was extracted using the Trizol reagent (Invitrogen, Carlsbad, CA, USA) and was reverse-transcribed into cDNA using revert aid first strand cDNA synthesis kit (Fermentas International Inc., Burlington, Canada) for qRT-PCR (MX 3005P, USA) in the presence of a fluorescent dye (SYBR Green I, Cwbio). GAPDH was used as internal controls and gene expression levels were calculated by the 2-ΔΔCt method.

2.8. Western blot

Protein extraction was obtained using a total protein extraction kit and subjected to electrophoresis on 10 % sodium dodecyl sulfate-polyacrylamide gels, which was finally transferred onto polyvinylidene difluoride (PVDF) membrane. The membrane was blocked with 5 % skimmed milk and then incubated with antibodies at 4 °C overnight. The membranes were washed with TPBS for three times, and subsequently incubated in TPBS containing fluorescent labelling second antibodies for 2 h at room temperature.

2.9. Luciferase reporter assay

The binding sites of MIR600HG to miR-1197 was predicted by the online software StarBase3.0. The Wild-type (Wt) MIR600HG and mutant-type (Mut) MIR600HG of binding sites were co-transfected with miR-1197 mimics and control mimics into PC cells. The relative luciferase activities were detected by the dual-luciferase reporter assay system.

3. Results

3.1. MIR600HG is expressed at low levels in PC tissues

By analyzing TCGA latest data, a volcano map was drawn and we selected differentially expressed genes of PC recurrence and non-recurrence patients (|logFC|>1, P adjust < 0.05) to generate a heat map. Among them, MIR600HG showed the significantly low expression in PC recurrence patients compared with the non-recurrence (Fig. 1A–C). These results suggested that MIR600HG might exert an effect in PC. To verify the above results, a total of 60 PC patients was selected and the expression of MIR600HG in the PC tissue was at lower levels, compared with that in the normal tissue (Fig. 1D).

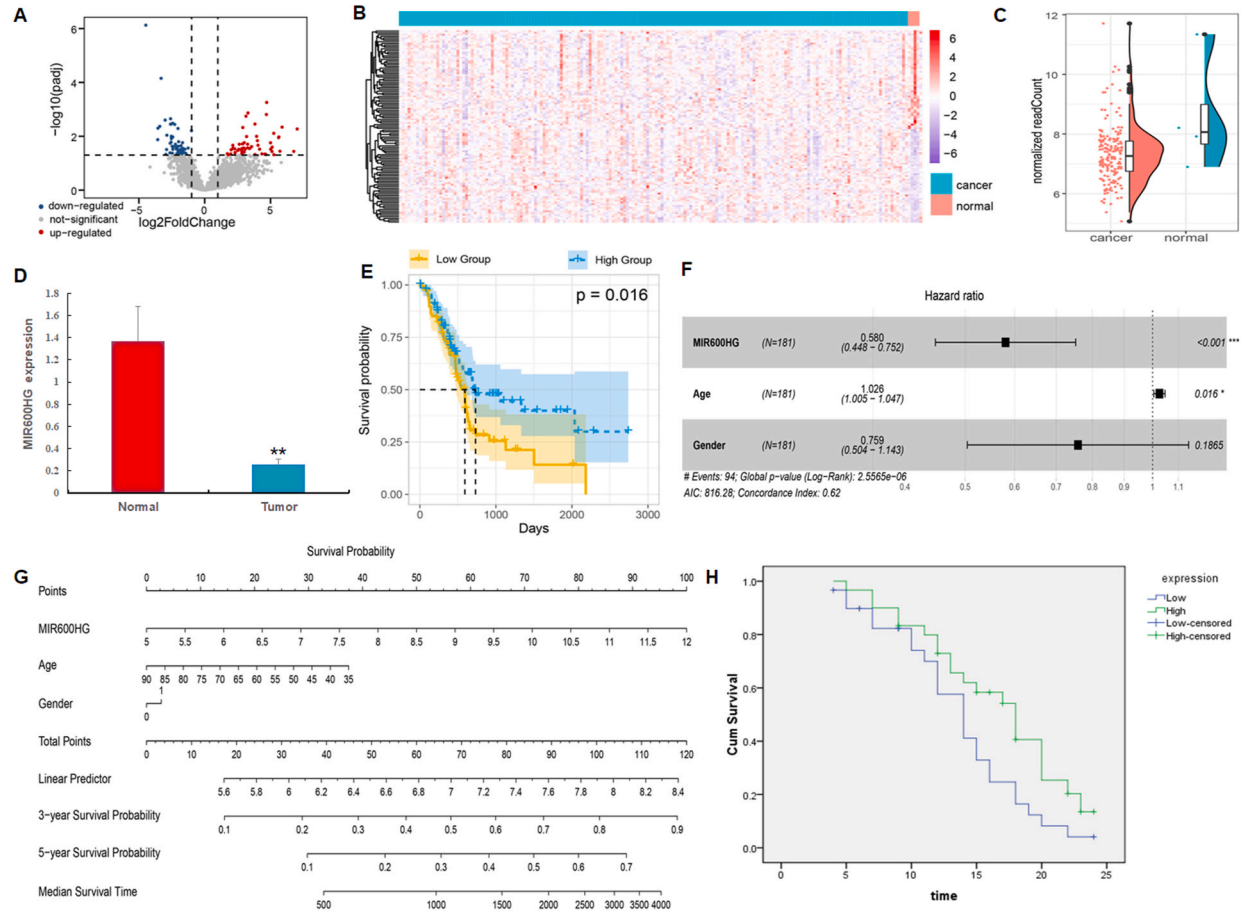

Fig. 1. Expression and prognostic value of MIR600HG in PC tissues and cell lines. A–C: Volcano map, heat map and violin map showing the TCGA differentially expressed genes and MIR600HG is expressed at low levels in PC tissues; D: The validation results for tissue differential expression of MIR600HG; E–G: Prognostic analysis diagram made by TCGA (KM survival curve, forest map and nomograms); H: Validated prognostic analysis diagram showing the relation between MIR600HG expression level and survival in PC patients. *$p < .05$, **$p < .01$, **$p < .001$.

3.2. Identification of significant differentially expressed MIR600HG for PC prognosis

The survival analysis of lncRNA was performed to verify whether MIR600HG expression is associated with the prognosis of PC. The patients were divided into two subgroups according to the median MIR600HG expression. The Kaplan-Meier (KM) survival curve

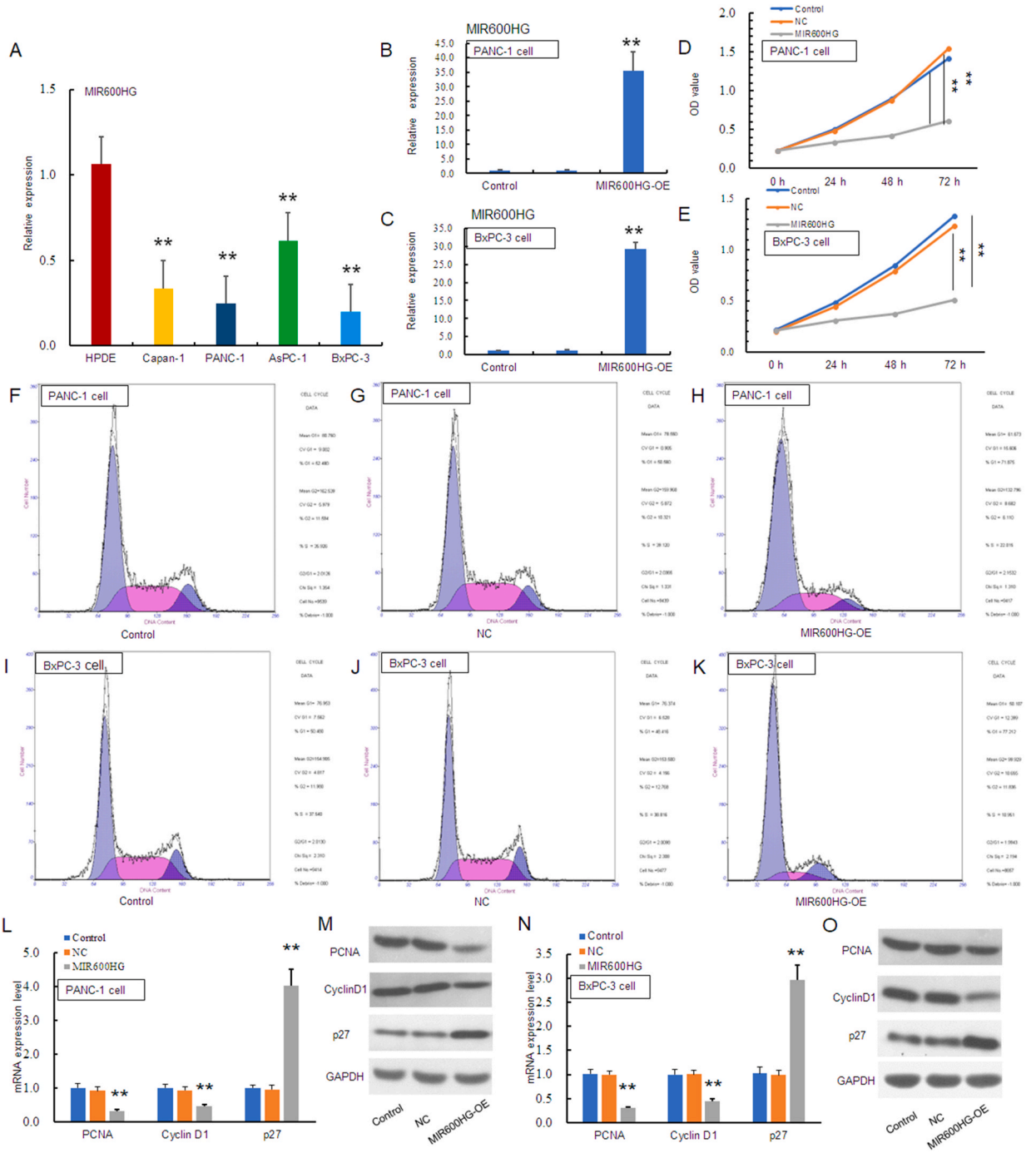

Fig. 2. MIR600HG regulated cell apoptosis and proliferation in vitro. A: MIR600HG expression in different cell lines; B–C: Verification of MIR600HG overexpression of cells transfected with MIR600HG overexpression plasmid (PANC-1 and BxPC-3 cells); D–E: Comparison of cell viability (PANC-1 and BxPC-3 cells); F–K: Changes of cell cycle (L–O) and expression of proliferation-related genes such as PCNA, CyclinD1 and P27 (H–I) after MIR600HG overexpression (PANC-1 and BxPC-3 cells). All experiments were repeated at least for three times and mean ± SD was used to represent the final result.

revealed that MIR600HG expression could successfully classify patients into good and poor prognosis groups, and high MIR600HG level was positively associated with overall survival rate (Fig. 1E). Later, the correlation between MIR600HG and overall survival was analyzed through multivariate Cox analysis. The forest map showed that MIR600HG expression could be used as an effective predictive factor (Figure 1F). Furthermore, 3- and 5-year survival rate could be predicted by nomograms. The patients were scored according to the corresponding indicators in the nomogram. Based on total points, the 3-year and 5-year survival rate of PC patients could be preliminarily estimated (Figure 1G). Last, we confirmed that the cumulative survival rate in MIR600HG high-expressed group significantly increased, compared with MIR600HG low-expressed group (Figure 1H).

3.3. MIR600HG regulated proliferation, invasion and migration of PC cells

The levels of MIR600HG in PC cell lines such as Capan-1, PANC-1, AsPC-1, and BxPC-3 were observed so as to investigate the effect of MIR600HG in PC cells. MIR600HG expression within PC cells dropped significantly, compared with normal HPDE cells (Fig. 2A). MIR600HG-OE plasmid was transfected into BxPC-3 and PANC-1 cells in order to upregulate MIR600HG levels, and MIR600HG expression in the MIR600HG-OE group increased remarkably in above cells (Fig. 2B and C). Compared with control group and empty plasmid group (NC group), cell viability was inhibited in MIR600HG-OE group (Fig. 2D and E). Cell apoptosis was enhanced (Fig. 2F–K). Moreover, MIR600HG reduced PCNA and CyclinD1 levels and increased P27 expression at the mRNA and protein level, which were considered to be associated with cell proliferation (Fig. 2L-O). Meanwhile, MIR600HG attenuated cell invasion and migration and down-regulated the expressions of MMP-9, MMP-7 and ICAM-1 at protein levels in PANC-1 (Fig. 3A–D) and BxPC-3 cell lines (Fig. 3E–H). Thus, MIR600HG suppressed PC cell growth, invasion, and migration.

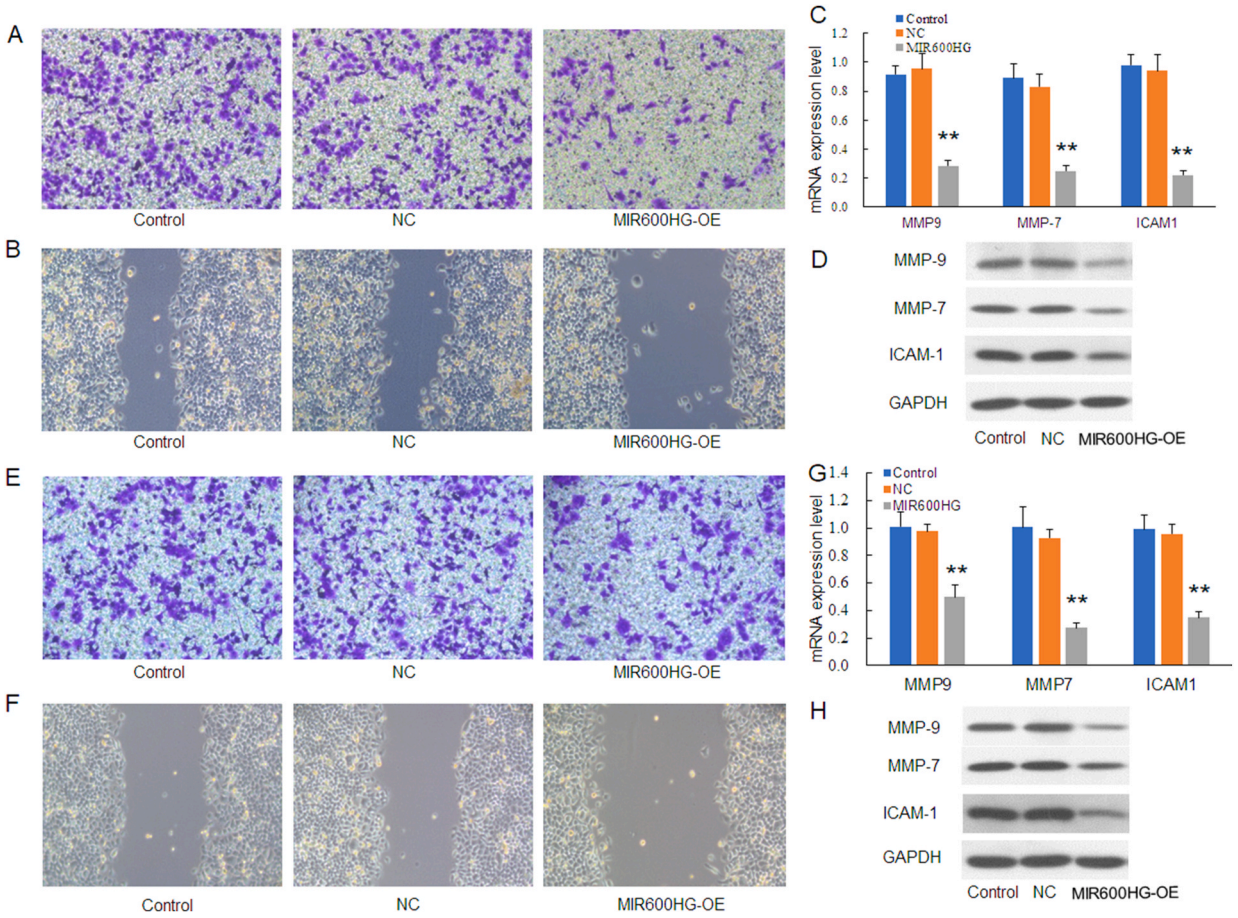

Fig. 3. MIR600HG regulated invasion and migration in vitro. A–D: Changes in invasion and migration ability, as well as invasion-related genes such as MMP-9, MMP-7 and ICAM-1 levels secondary to MIR600HG overexpression in PANC-1 cells. E–H: Changes in invasion and migration ability, as well as invasion-related genes such as MMP-9, MMP-7 and ICAM-1 levels secondary to MIR600HG overexpression in BxPC-3 cells). $*p < .05$, $**p < .01$, $**p < .001$. All experiments were repeated at least for three times and mean ± SD was used to represent the final result.

3.4. MIR600HG played a part of sponging miR-1197 in PC

We predicted miR-1197 as the candidate MIR600HG target on the StarBase website. Then, MIR600HG was found to have a binding site for miR-1197 (Fig. 4A). The RNA levels of miR-1197 increased in PC tissues and different PC cells (Fig. 4B–C). MIR600HG was negatively associated with miR-1197 in PC tissues through Pearson correlation analysis (Fig. 4D). Accordingly, the miR-1197 mRNA expression was dramatically decreased in MIR600HG over-expressed cells such as PANC-1 and BxTC-3, relative to that in control and NC group (Fig. 4E and F). As observed, miR-1197 mimic remarkably decreased the MIR600HG-WT reporter luciferase activity, but the difference was not significant compared with the MIR600HG-Mut reporter (Fig. 4G). All these results indicated that MIR600HG was important in regulating miR-1197.

3.5. PITPNM3 mRNA as miR-1197 target gene in PC

Based on the Targetscan and the miRDB database, we predicted PITPNM3 as the candidate miR-1197 target. Then, PITPNM3 had a binding site for miR-1197 through bioinformatics analysis (Fig. 5A). PITPNM3 was down-regulated in PC tissues (Fig. 5B). In order to clarify the correlation among PITPNM3, MIR600HG and miR-1197, Pearson correlation analysis indicated that MIR600HG and PITPNM3 expression levels showed a positive correlation in PC tissues (Fig. 5C). Nevertheless, the levels of miR-1197 and PITPNM3 mRNA in PC were negatively correlated (Fig. 5D). Similarly, PITPNM3 expressions were dropped in different PC cell lines, compared with normal cell lines (Fig. 5E). Western-blot and qRT-PCR also illustrated that PITPNM3 expressions were dramatically increased in the miR-1197 inhibitor group (miR-1197 group), compared with that in NC inhibitor group (NC group) and control group (Fig. 5F–I). In order to test the correlation of PITPNM3 and miR-1197, a luciferase reporter assay was carried out. We found that miR-1197 mimic remarkably decreased the PITPNM3-WT reporter luciferase activity, but the difference was not significant compared with PITPNM3-Mut reporter (Fig. 5J). Thus, PITPNM3 acted as the miR-1197 Target gene in PC.

3.6. PITPNM3 was involved in MIR600HG/miR-1197 - mediated Inhibition of PC Progression

Whether MIR600HG regulates PITPNM3 expression through acting on miR-1197 still needs to be further studied. It indicated that PITPNM3 expression showed a corresponding increase by transfecting with MIR600HG-OE plasmid, but this increase partially abolished in cells co-transfected with both MIR600HG-OE plasmid and miR-1197 mimics (Fig. 6A and B). Correspondingly, miR-1197 mimics inhibited the role of MIR600HG-OE in reducing PC cell invasion and migration (Fig. 6C and D). MIR600HG was found to reduce

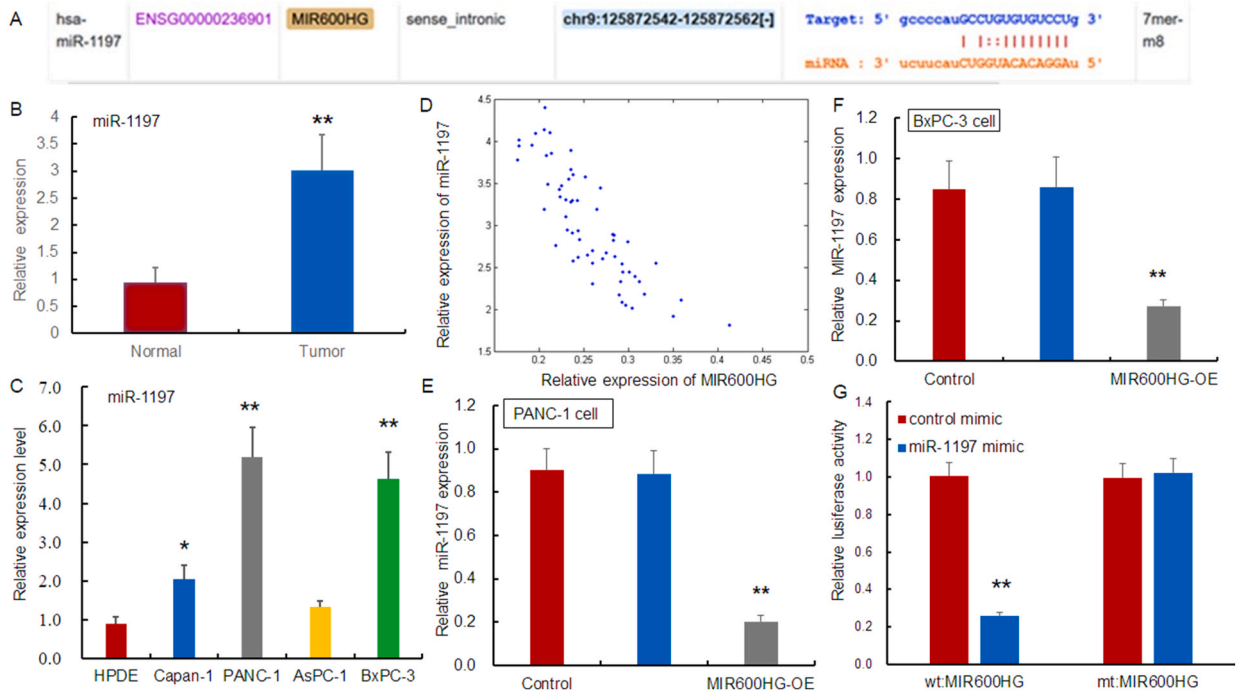

Fig. 4. Relationship between MIR600HG and miR-1197. A: miR-1197 was predicted as a potential target of MIR600HG by the starBase website; B–C: Expression level of miR-1197 in tissues and cell lines; D: The expression levels of MIR600HG and miR-1197 in tissues were negatively correlated; E–F: Effect of MIR600HG overexpression on miR-1197 in PANC-1 and BxPC-3 cells; G: Binding of MIR600HG to miR-1197 verified by dual-luciferase reporter gene assay. *$p < .05$, **$p < .01$, **$p < .001$. All experiments were repeated at least for three times and mean ± SD was used to represent the final result.

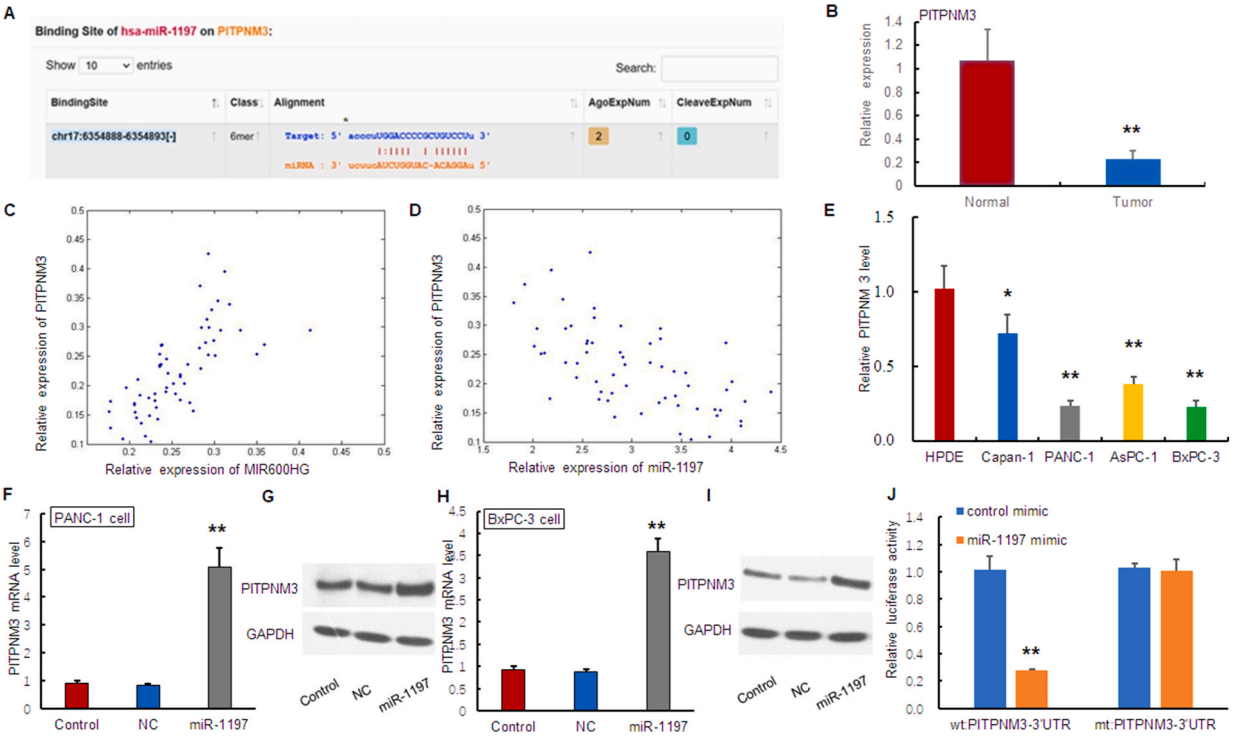

Fig. 5. Relationship between PITPNM3 mRNA and miR-1197. A: PITPNM3 mRNA predicted results by the Targetscan and miRDB database; B: Expression level of PITPNM3 mRNA in tissues; C: The expression levels of MIR600HG and PITPNM3 mRNA in tissues were positively correlated; D: The expression levels of miR-1197 and PITPNM3 mRNA in tissues were negatively correlated E: Expression level of PITPNM3 mRNA in different cell lines; F–I: Effect of miR-1197 inhibitors on PITPNM3 at mRNA and protein levels in PANC-1 and BxTC-3 lines; J: Binding of PITPNM3 mRNA to miR-1197 verified by dual-luciferase reporter gene assay. $*p < .05$, $**p < .01$, $**p < .001$. All experiments were repeated at least for three times and mean ± SD was used to represent the final result.

the levels PCNA, CyclinD1, MMP-9, MMP-7 as well as ICAM-1, and to increase P27 expression in vitro. Nevertheless, miR-1197 mimics attenuated the effect of MIR600HG-OE on above mRNA and proteins associated with cell proliferation, invasion and migration (Fig. 6E and F). Collectively, above results suggested that MIR600HG acted as a ceRNA which impeded PC progress by regulating miR-1197/PITPNM3 axis.

4. Discussion

PC has received more and more attention in consideration of its rather poor prognosis [10]. The molecular heterogeneity of PC might demand as much diversified treatment approaches based on individual tumor characteristics. Emerging studies have shown that lncRNAs play an important role in the initiation and development of cancers including PC [11]. Many scientists have found that lncRNAs exert the biological effects in epigenetics, at the histone modification, transcriptional and posttranscriptional levels [12,13]. A series of studies have elucidated the extraordinary effect of lncRNAs in a wide range of diseases, such as cardiovascular diseases, diabetes, neurodegenerative diseases and cancers [14].

Although a large number of lncRNAs have been identified in the human genome, only a very few have been experimentally validated and functionally annotated in PC. LncRNAs, which can interact with microRNAs, are associated with genomic stability. However, the regulatory relationship of lncRNAs and genomic stability has not been completely investigated in PC. LncRNAs have been confirmed to sponge miRNAs as ceRNA to modulate cancer progression [15,16]. For example, LINC00514 acted as a ceRNA of miR-28-5p to increase Rap1b level in the stage of PC progression [17]. MIR600HG has been previously validated in colorectal cancer [8,18]. Xiao et al. considered that MIR600HG in the ceRNA network, immune - associated T cell and macrophages were all possibly associated with the pancreatic adenocarcinoma prognosis [19]. It was also found that MIR600HG and other four lncRNAs might act as the potential biomarkers in pancreatic ductal adenocarcinoma [20]. Nevertheless, it still needs further study to focus on the function of MIR600HG in PC.

We found that the level of MIR600HG dropped, relative to the normal PC tissue and cells, and that reduced level of MIR600HG was associated with PC progression and poor survival. Tian et al. found that the risk score was negatively associated with the levels of MIR600HG in PC [21], which was in accordance with our result. It also showed that MIR600HG inhibited cell proliferation, migration and invasion, and induced cell apoptosis in PC. Thus, MIR600HG might play an anti-cancer role in PC. The underlying molecule mechanisms, however, were still not clear. More experimental studies are still needed to assess the value of MIR600HG in PC tissues

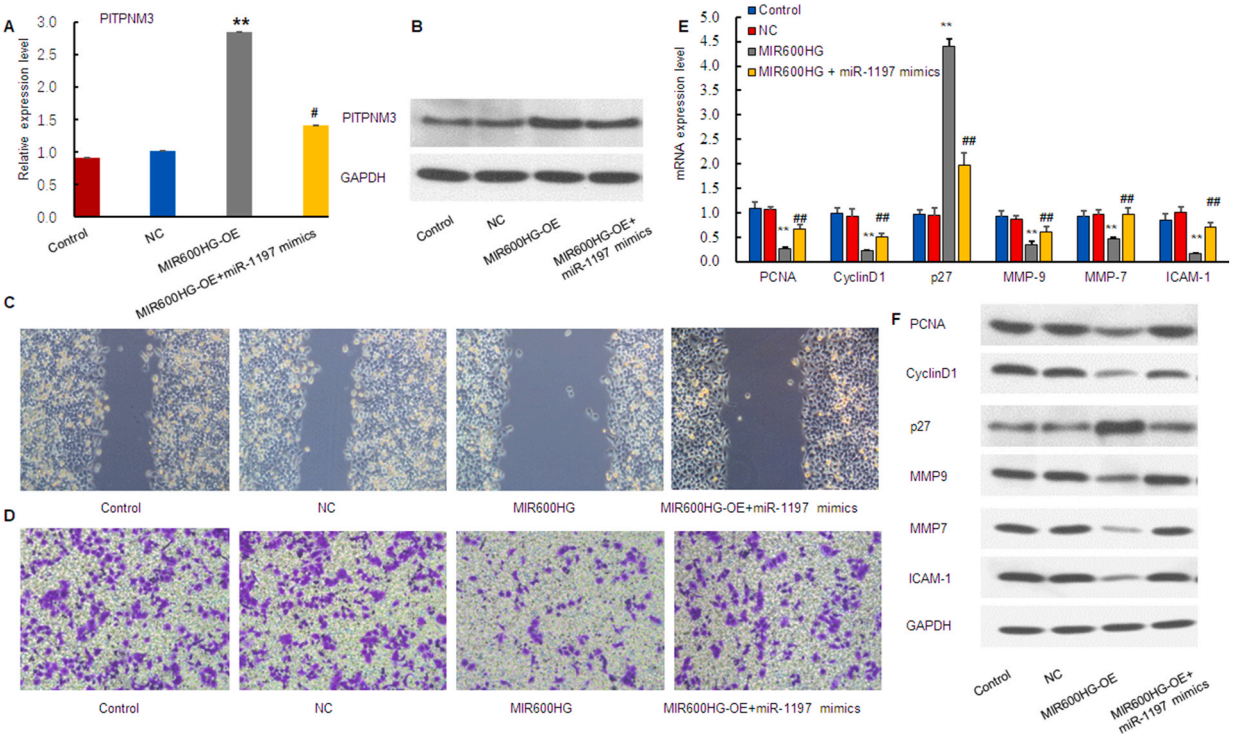

Fig. 6. Effect of MIR600HG/miR-1197 axis on pancreatic cancer cells. A–B: The effect of MIR600HG overexpression on PITPNM3 mRNA and protein in cells (control group, NC group, MIR600HG-OE group, MIR600HG-OE + miR-1197 mimic group); C–D: Effects of MIR600HG overexpression on cell activity, migration and invasion in control group, NC group, MIR600HG-OE group, and MIR600HG-OE + miR-1197 mimic group); E–F: Effects of MIR600HG overexpression on mRNAs and protein related to cell proliferation, migration and invasion in control group, NC group, MIR600HG-OE group, and MIR600HG-OE + miR-1197 mimic group (* vs. NC or control group, $^{*}p < .05$, $^{**}p < .01$, $^{**}p < .001$; $^{\#}$ vs. MIR600HG-OE group, $^{\#}p < .05$, $^{\#\#}p < .01$, $^{\#\#}p < .001$). All experiments were repeated at least for three times and mean ± SD was used to represent the final result.

and cells.

Emerging evidence has proven that lncRNA could act on ceRNA to influence cancer prognosis [22]. For instance, CYTOR could facilitate PC development through sponging miR-205-5p [23]. The lncRNA 00976 was confirmed to act as a ceRNA of miR-137 in PC development [24]. In hepatocellular carcinoma, miR-1197 was found to be the potential target of Circ_0004018 and could suppresses the proliferation and infiltration of tumor cells [25]. In this article, we considered miR-1197 to be the target of MIR600HG, and confirmed the interaction between MIR600HG and miR-1197. MIR600HG-OE down-regulated miR-1197 expression. Thus, MIR600HG was confirmed to act as a ceRNA of miR-1197 in the development of PC.

PITPNM3 was associated with various cancers, including hepatocellular carcinoma, breast cancer, and intrahepatic cholangiocarcinoma [26–28]. Nevertheless, there is a limited amount of study of PITPNM3 in PC [29–31]. In our study, PITPNM3 mRNA was a direct target of miR-1197. The luciferase reporter assay tested the binding relationship between PITPNM3 and miR-1197. A positive correlation between PITPNM3 and MIR600HG, and a negative relationship between PITPNM3 and miR-1197 were found. PITPNM3 mRNA and protein expression presented a rise by transfection with MIR600HG-OE plasmid, which was partially abolished in cells transfected with over-expressed plasmid and miR-1197 mimics. The following study found that MIR600HG exerted an anti-cancer effect through inhibiting miR-1197, which was found to accelerate the PC progression. Therefore, there might be a need to develop an effective therapy of PITPNM3 agonist for the treatment of PC patients, and some targeted therapy might offer hope for PC treatment.

Nevertheless, there are limitations to this study. The different PC pathological types and clinical heterogeneity might lead to some bias. The potential role of MIR600HG, miR-1197 and PITPNM3 as diagnostic and prognostic markers needs to be further analyzed. Therefore, animal model experiments should be applied with the hope that this approach would help completely verify the effect of MIR600HG/miR-1197/PITPNM3 *in vivo* and eliminate the influence of individual difference and other confounding factors. In addition to its ceRNA function, there could be other potential mechanisms through which MIR600HG regulates PC progression. The specific mechanism of MIR600HG thus still needs to be further explored.

In a word, the present work suggested a novel mechanism for PC progression that MIR600HG exerted the anti-tumor effect by sponging miR-1197 and raising the expression of PITPNM3. It indicates the value of MIR600HG/miR-1197/PITPNM3 axis in the development of PC and might be a valuable target for the PC prognosis and therapies in the future.

Data availability

All data associated with my study was not deposited into a publicly available repository, but they will be made available on request.

Ethics approval

The ethics was approved by the Human Research Ethics Committee of Fourth Affiliated Hospital of Hebei Medical University (2021KY245). All the patients involved have signed informed consent forms.

CRediT authorship contribution statement

Baoming Yang: Writing – review & editing, Writing – original draft, Formal analysis, Data curation, Conceptualization. **Zhikai Jiao:** Supervision. **Ningning Feng:** Supervision. **Yueshan Zhang:** Supervision. **Shunxiang Wang:** Resources, Funding acquisition.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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