MEDICAL SCIENCE MONITOR

LAB/IN VITRO RESEARCH

e-ISSN 1643-3750
© Med Sci Monit, 2019; 25: 7509-7517
DOI: 10.12659/MSM.917038

Received: 2019.04.18
Accepted: 2019.06.21
Published: 2019.10.07

miRNA-339-5p Plays an Important Role in Invasion and Migration of Pancreatic Cancer Cells

Authors' Contribution:
Study Design A
Data Collection B
Statistical Analysis C
Data Interpretation D
Manuscript Preparation E
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Source of support: Youth Fund of National Natural Science Foundation of China (programme no.: 81502505)

Background: This study aimed to investigate the role of miRNA-339-5p in pancreatic cancer cell invasion and migration.

Material/Methods: The differences between exosomal miRNAs of PANC02 and PANC02-H7 were studied by microarray analysis. We measured miRNA-339-5p expression in different groups; differences in cell invasion and migration were evaluated using the Transwell and wound healing assays and expression of relative proteins (E-cadherin, vimentin and ZNF689) was measured by WB assay. The correlation between miRNA-339-5p and ZNF689 expression was evaluated by luciferase reporter gene assay.

Results: Compared with PANC02 exosome, microarray analysis indicated that miRNA-339-5p mRNA expression was significantly suppressed (P<0.001) in the PANC02-H7 exosome. Supplementation with miR-339-5p mimics led to a significant decrease in the invasion cell number and wound healing rate (P<0.001), with significantly enhanced E-cadherin expression and suppressed vimentin expression (P<0.001). However, transfection of a miR-339-5p inhibitor led to a significant increase in the invasion cell number and wound healing rate (P<0.001), with significantly suppressed E-cadherin expression and increased vimentin expression (P<0.001). Luciferase reporter gene assay demonstrated ZNF689 gene to be the target of miR-339-5p in the PANC02-H7 cell. With miR-339-5p and ZNF689 transfection, the invasion cell number and wound healing rate were significantly increased compared with those in the miR-339-5p group (P<0.001), with significantly increased expression of ZNF689 and vimentin and suppressed E-cadherin expression (P<0.001).

Conclusions: miR-339-5p suppresses the invasion and migration of pancreatic cancer cells via direct regulation of ZNF689 *in vitro*.

MeSH Keywords: MicroRNAs • Neoplasm Invasiveness

Full-text PDF: https://www.medscimonit.com/abstract/index/idArt/917038

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Background

Pancreatic cancer is a malignant tumor of the digestive system with extremely high mortality. Its incidence has been increasing over the years, thereby posing a serious threat to human health. The most common type of pancreatic cancer is pancreatic ductal adenocarcinoma (PDAC), accounting for 80–90% of all pancreatic tumors. Owing to its asymptomatic nature, PDAC is mostly diagnosed at an advanced stage. PDAC has a poor prognosis, with a median survival time of only 3–5 months, and the 1-year survival rate is <10%. Distant metastasis of cancer cells primarily contributes to the low survival rate of pancreatic cancer [1]. To date, CA19-9 is the only FDA-approved biomarker for PDAC screening; however, its efficiency is low. Significant advances in pancreatic cancer research have allowed the identification and development of a large number of markers for the prognosis of PDAC. However, these markers are not yet approved for use in clinical practice. Therefore, the discovery of novel potential prognostic markers of PDAC for early intervention and prognosis is critical.

miRNAs are a type of negative regulators of gene expression. They are abnormally expressed in various tumors and promote the occurrence and development of tumors by regulating their corresponding target genes, which makes them the focus of research in tumor diagnosis and targeted therapy [2,3]. Gene-targeted therapy, a new strategy for tumor treatment, can inhibit gene expression in tumor cells via specific targets genes, thereby inhibiting tumor progression [4]. Zinc finger proteins (ZNFs) are a class of proteins that bind to Zn^{2+} and selectively bind to specific target structures, thereby playing an important role in the regulation of gene expression, cell differentiation, and embryonic development [5]. ZNF689 is a ZNF member that is highly expressed in tumor cells [6]. Studies have reported that miR-339-5p can target and regulate ZNF689 [7].

In the present study, abnormally expressed genes in PANC02 and PANC02-H7 cell lines were screened by miRNA microarray analysis. The expression of miR-339-5p was observed to be significantly decreased in PANC02-H7. We speculate that miR-339-5p inhibits the invasion and migration of pancreatic cancer cells by regulating ZNF689.

Material and Methods

Cells

The normal mouse pancreatic cancer cell line PANC02 and the highly metastatic mouse pancreatic cancer cell line PANC02-H7 were purchased from the American Type Culture Collection.

RNA extraction and microarray analysis

Total RNA was extracted from the exosome of PANC02 and PANC02-H7 cells using TRIzol reagent (Invitrogen, USA) according to manufacturer's instructions. The purity and concentration of extracted RNAs were determined by NanoDrop2000/2000C (Thermo Corporation, USA), with acceptable purity indicated by an absorbance ratio of A260/A280 ≥1.8 or A260/A230 ≥1.5. The extracted RNA samples were stored at -80°C. Differentially expressed (DE) genes in PANC02 and PANC02-H7 cells were identified using miRNA microarrays. Small RNAs were pre-enriched using Nanoseplook (Pall Corporation, USA) using 2.0 µg total RNA samples and labelled with miRNA ULSTM labeling kit (Kreatech, Netherlands). Labelled miRNAs were hybridized with the miRNA chip, followed by washing at 37°C for 16 h. The chip was then scanned using an Axon 400B scanner (Sunnyvale, USA).

Database analysis

miRNA and mRNA expression data were retrieved from the Cancer Genome Atlas database (*https://cancergenome.nih.gov/*) and DE genes in the database were searched using the R package. The expression of miRNA-339-5p in PANC02 and PANC02-H7 was quantitatively analyzed.

Cell culture and transfection

Mouse PANC02-H7 cells were cultured in an incubator at 37°C with 5% CO_2 in DMEM supplemented with 10% fetal bovine serum (FBS) and 1% penicillin/streptomycin. miR-339-5p mimics, miR-339-5p inhibitor (Suzhou GenePharma, China), and ZNF689 were transfected into PANC02-H7 cells by Lipofectamine™ 2000 as per the manufacturer's instructions. Cells were cultured for 48 h and collected for subsequent assays. Cell morphology in each group was observed using an inverted microscope (100×).

Real-time fluorescence quantitative polymerase chain reaction (RT-qPCR)

Total RNA was extracted from placental tissues using the TRIzol one-step method according to the instructions provided with the RNAiso™ Plus reagent (TaKaRa Bio, Dalian, China). Reverse transcription was performed using the PrimeScript RT reagent kit (TaKaRa Bio, Dalian, China) according to manufacturer's instructions. The following primers were designed and synthesized by TaKaRa Bio (Dalian, China):
U6: F: 5'-GCTTCGGCAGCACATATACTAAAAT-3';
R: 5'-CGCTTCACGAATTTGCGTGTCAT-3'. MiR-339-5p:
F: 5'-ACACTCCAGCTGCGGTCCCTGTCCTCCAGGAG-3';
R: 5'-TGGTGTCGTGGAGTCG-3'.
For RT-qPCR reaction, cDNA (0.8 µL) template was combined with 0.4 µL of each primer (10 µmol/L each), 5 µl SYBR Premix

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Ex TaqIM II (2×) (Takara, Dalian, China), and 3.4 µl DDH_2O to a final volume of 10 µL. PCR cycling was performed as follows: initial denaturation at 95°C for 30 s, followed by 40 cycles of denaturation at 95°C for 5 s, and annealing at 60°C for 30 s. At the end of the reaction, the amplification and melting curves were recorded to determine the specificity of sample amplification. The CT value of each sample was obtained based on the PCR amplification curve, and the relative expression level of miR-339-5p was calculated using the $2^{-\Delta\Delta CT}$ method and normalized using U6 as an internal reference.

Transwell assay

Matrigel in the chambers was rehydrated. PANC02-H7 cells were re-suspended with FBS-supplemented DMEM at a density of 1×10^6/ml, and 200 µl of cell suspension was loaded into the upper chamber of each test group. DMEM (containing 500 µL FBS) was added to each lower chamber. The cells were cultured for 24 h in an incubator, and the medium was subsequently discarded. Cells were washed and those on the bottom of the lower chamber were stained with 0.5% crystal violet solution. The number of cells in the same field was counted using a microscope.

Wound healing assay

Cell migration was measured using a scratch assay. Briefly, cell suspension at a density of 1×10^6/mL was plated in a 6-well plate and cultured overnight until a monolayer was formed. A straight scratch was introduced on the cell monolayer with a 10-µL sterile pipette tip. The detached cells were washed 3 times with PBS and microscopic images were recorded at 0 h. Subsequently, serum-free medium was added and cells were cultured for another 24 h, followed by microscopic imaging. The rate of wound healing was calculated as: wound healing rate=(cell spacing at 0 h -cell spacing at 24 h)/cell spacing at 0 h×100%.

Luciferase reporter gene assay

To validate the microRNA-binding sequence of miR-339-5p, fragments containing wild-type or mutated ZNF689-3'-untranslated region (3'-UTR) of the predicted binding site (ZNF689wt or HZNF689 mut) were cloned into the GV272 vector. Upon confirmation by DNA sequencing, these constructs (with or without miR-339-5p mimic) were transfected into PANC02-H7 cells by Lipofectamine™ 2000 according to manufacturer's instructions. Cells were collected at 48 h after transfection and analyzed using a dual luciferase reporter system.

Western blot

Cells in each group were collected and washed twice with PBS. Cell lysis buffer was added to cells and the mixture was kept on ice for 15 min. Loading buffer was added to the cell lysates and the mixture was placed for 10 min in a water bath with boiling water. The total protein concentration was determined using the BCA kit and equal amounts (20 µg) of protein sample were used for separation on SDS-PAGE. After electrophoresis, proteins were transferred to a PVDF membrane. The membrane was blocked with 5% skim milk solution and then incubated overnight at 4°C with anti-ZNF689, E-cadherin, and vimentin antibodies (1: 1000). After washing, the membranes were incubated with IgG-HRP (1: 5000) secondary antibody. The target protein was detected using an ECL system (Millipore, Germany) and the relative expression level was calculated by counting the grey value using Image J software with GAPDH as an internal reference. At least 3 independent experiments were performed.

Statistical analysis

Statistical analysis was performed using SPSS 17.0 software. Differences in migration and invasion ability and mRNA and protein expression between the treatment and the control groups were analyzed by *t* test, one-way ANOVA, post hoc test, and two-way ANOVA. A P value of <0.05 was considered statistically significant.

Results

Expression levels of miR-339-5p in PANC02 and PANC02-H7 cells

miRNA microarray analysis revealed 16 DE genes in PANC02-H7 cells; among these, 8 miRNAs were up-regulated and 8 were down-regulated. miR-339-5p was the most clearly identifiable DE gene among the 16 DE genes (P<0.001, Figure 1).

Effect of miR-339-5p on PANC02-H7 cells

The co-transfection of miR-339-5p led to a significant increase in the expression of miR-339-5p mRNA in the miR-339-5p mimics group compared with that in the scramble group (P<0.001, Figure 2A). No significant differences in cell morphology were observed between the 2 groups (Figure 2B). The number of invasive cells (Figure 2C) and wound healing rate (Figure 2D) were significantly decreased in the miR-339-5p mimics group compared with those in the scramble group (P<0.001). Western blot analysis showed that the expression of E-cadherin and vimentin was significantly increased and decreased, respectively, in the miR-339-5p group (P<0.001, Figure 2E).

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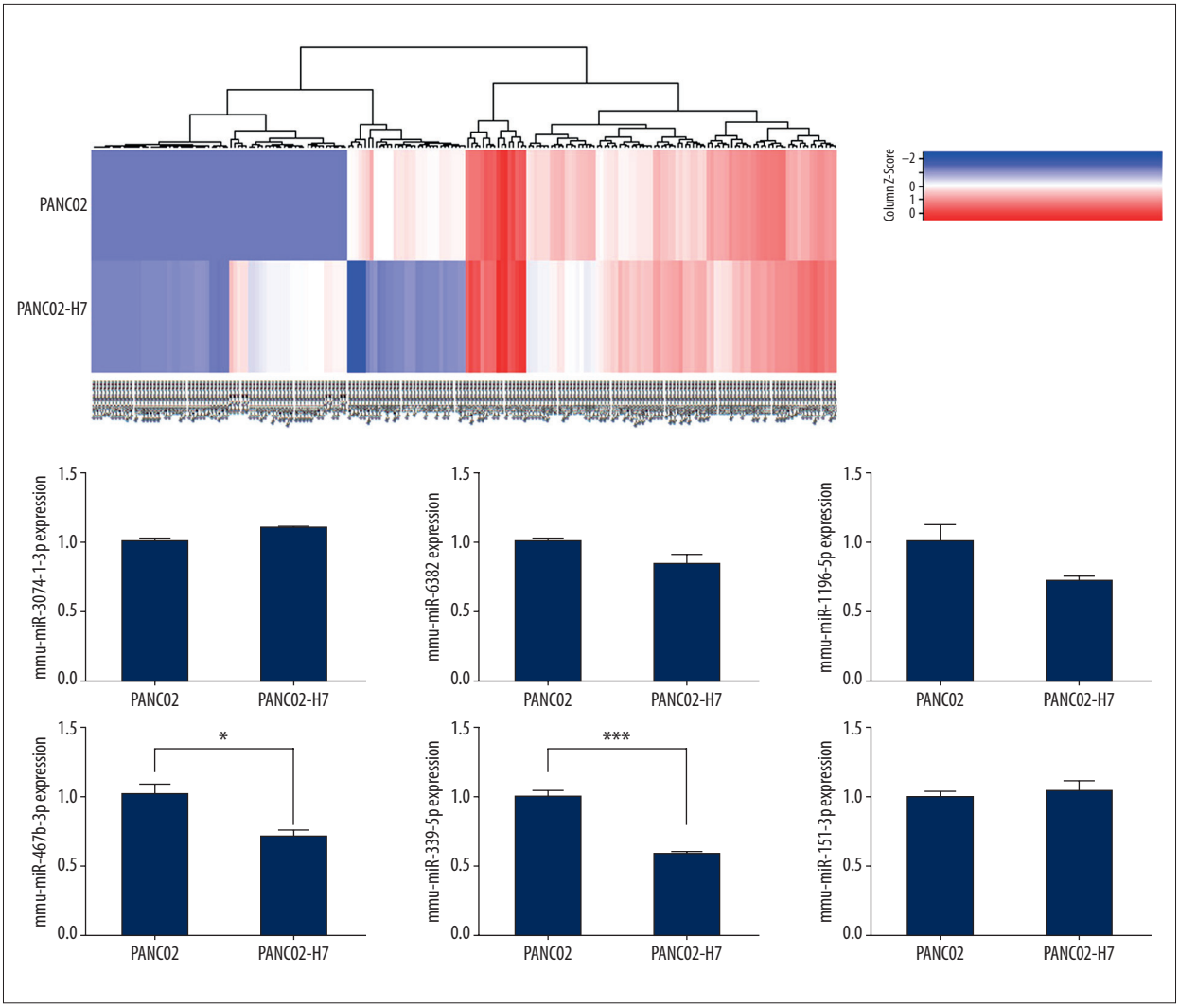

Figure 1. Expression level of miRNAs and heat-map in PANC02 and PANC02-H7 cells. * P<0.05, ** P<0.01, *** P<0.001, compared with PANC02 cells.

Effect of miR-339-5p blockade on PANC02-H7 cells

The co-transfection of miR-339-5p inhibitor led to a significant reduction in miR-339-5p mRNA expression in the miR-339-5p inhibitor transfection group compared with that in the scramble group (P<0.001, Figure 3A). No significant differences in cell morphology were observed between the 2 groups (Figure 3B). The number of invasive cells (Figure 3C) and wound healing rate (Figure 3D) were significantly increased in the miR-339-5p inhibitor group compared with those in the scramble group (P<0.001). Western blot analysis showed that the expression of E-cadherin and vimentin was significantly decreased and increased, respectively, in the miR-339-5p inhibitor group (P<0.001, Figure 3E).

miR-339-5p directly targeted ZNF689

It was predicted that miR-339-5p targets and regulates the translation of ZNF689. The luciferase activity in cells transfected with the wt ZNF689-3'-UTR construct was reduced by miR-339-5p and the difference between the 2 groups was statistically significant (P<0.001, Figure 4A). The luciferase activity in cells transfected with Mut ZNF689 3'-UTR construct remained unchanged, indicating that miR-339-5p negatively regulated the expression of ZNF689 gene by directly binding to the 3'-UTR of ZNF689 (Figure 4A). Western blot analysis showed that miR-339-5p reduced the expression of ZNF689 protein in cells transfected with wt ZNF689-3'-UTR (P<0.001, Figure 4B). No statistically significant difference in ZNF689 protein expression was noted in Mut ZNF689-3'-UTR transfected cells between the miR-NC group and the miR-339-5p group.

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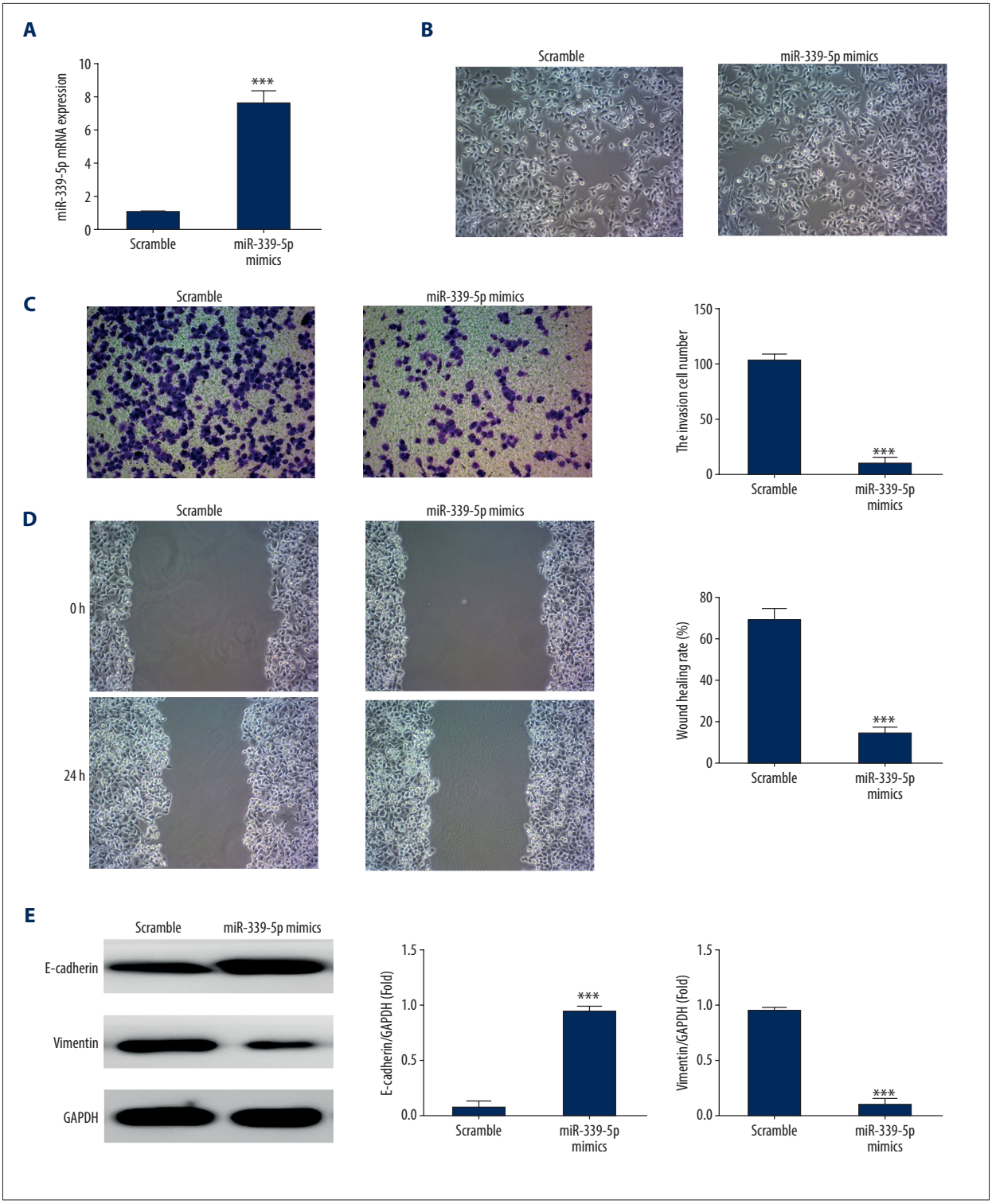

Figure 2. miR-339-5p overexpression affected PANC02-H7 cell invasion and migration and relative proteins expression. (**A**) miR-339-5p mRNA expression in different groups as shown by RT-qPCR. *** P<0.001, compared with scramble group. (**B**) The PANC02-H7 cell morphology of different groups. (**C**) The invasion cell number of difference groups by Transwell assay. *** P<0.001, compared with scramble group. (**D**) The wound healing rate of different groups as shown by wound healing assay. *** P<0.001, compared with scramble group. (**E**) The relative proteins expression of different groups as shown by WB assay. *** P<0.001, compared with Scramble group.

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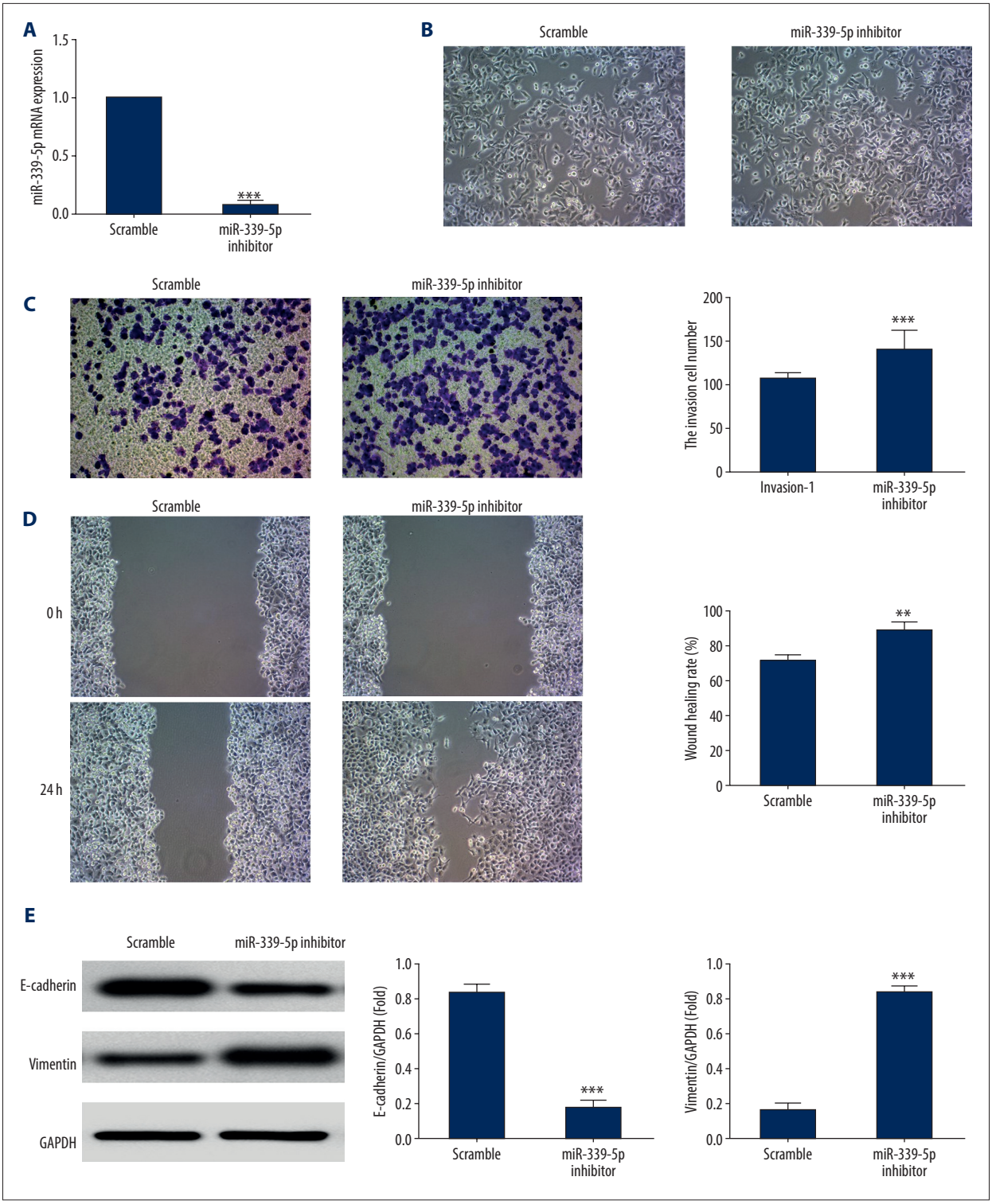

Figure 3. miR-339-5p inhibitor affected PANC02-H7 cell invasion and migration and relative proteins expression. (**A**) miR-339-5p mRNA expression in different groups as shown by RT-qPCR. *** P<0.001, compared with scramble group. (**B**) The PANC02-H7 cell morphology of different groups. (**C**) The invasion cell number of different groups as shown by Transwell assay. *** P<0.001, compared with scramble group. (**D**) The wound healing rate of different groups as shown by wound healing assay. *** P<0.001, compared with scramble group. (**E**) The relative proteins expression of difference groups as shown by WB assay. *** P<0.001, compared with Scramble group.

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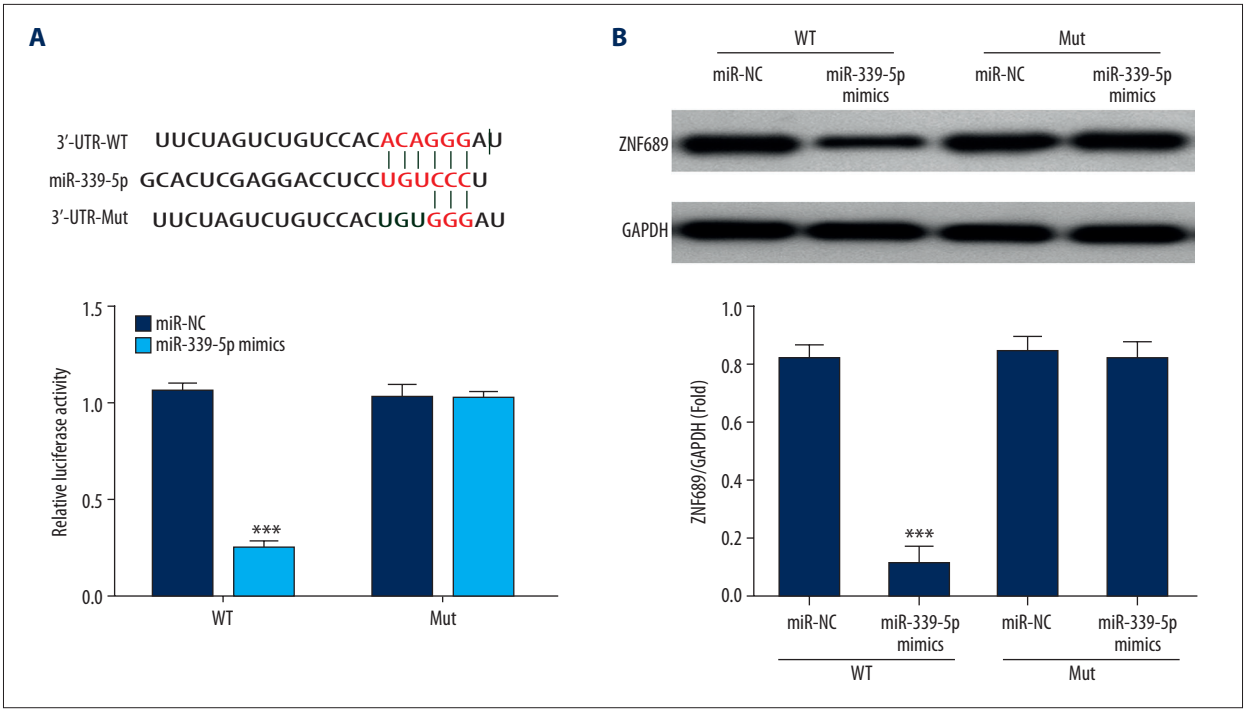

Figure 4. The correlation between miR-339-5p and ZNF689 and ZNF689 protein expression in PANC02-H7 cells. (**A**) The correlation between miR-339-5p and ZNF689 as shown by Luciferase reporter gene assay. *** P<0.001, compared with miR-NC group. (**B**) ZNF689 protein expression by WB assay. *** P<0.001, compared with miR-NC group.

Effects of miR-339-5p and ZNF689 on PANC02-H7 cells

The co-transfection of miR-339-5p or ZNF689 led to a significant increase in the expression of miR-339-5p mRNA in the miR-339-5p mimics and miR-339-5p mimics + ZNF689 groups compared with that in the scramble group (P<0.001, Figure 5A), and no significant differences in cell morphology were observed between the 2 groups (Figure 5B). The number of invasive cells (Figure 5C) and wound healing rate (Figure 5D) were significantly decreased in the miR-339-5p transfection and miR-339-5p+ZNF689 co-transfection groups compared with those in the scramble group (P<0.001). Western blot analysis revealed that, compared with that in the scramble group, the expression of E-cadherin was significantly increased, whereas that of ZNF689 and vimentin was significantly decreased in the miR-339-5p group (P<0.001, Figure 5E). In the miR-339-5p mimics group, the expression of E-cadherin was significantly decreased, whereas that of ZNF689 and vimentin was significantly increased compared with that in the scramble group (P<0.001, Figure 5E).

Discussion

Compared with other types of cancer, the mortality rates associated with pancreatic cancer rank sixth in China. Pancreatic cancer is expected to become the second leading cause of death as the incidence and associated mortality is gradually increasing [8]. Unfortunately, only small advances have been achieved regarding the diagnosis and treatment of pancreatic cancer in recent years [9]. As advanced pancreatic cancer cannot be completely cured, the associated prognosis is extremely poor and the 5-year survival rate is less than 5%. Distant metastasis of pancreatic cancer is the primary factor contributing to the high recurrence and mortality associated with this disease [10].

miR-339-5p is confirmed to function as a tumor suppressor in various cancers. The low expression of miR-339-5p is reportedly associated with increased metastasis and advanced clinical stages of breast cancer [11]. Weber et al. [12] showed significant differences in the expression of miR-339-5p between melanoma cells and normal melanocytes. Other studies have also shown that miR-339-5p effectively inhibits the activity of melanoma cells, and its overexpression effectively inhibits their invasiveness. miR-339-5p also plays an important role in regulating the metastasis of ovarian cancer [13]. In the present study, microarray analysis of the normal mouse pancreatic cancer cell line PANC02 and the highly metastatic mouse pancreatic cancer cell line PANC02-H7 demonstrated low expression levels of miR-339-5p in the PANC02-H7 cell line. On this basis, we reasoned that the inhibition of miR-339-5p expression may be a key factor for the enhanced invasion and migration of pancreatic cancer cells. To validate this hypothesis,

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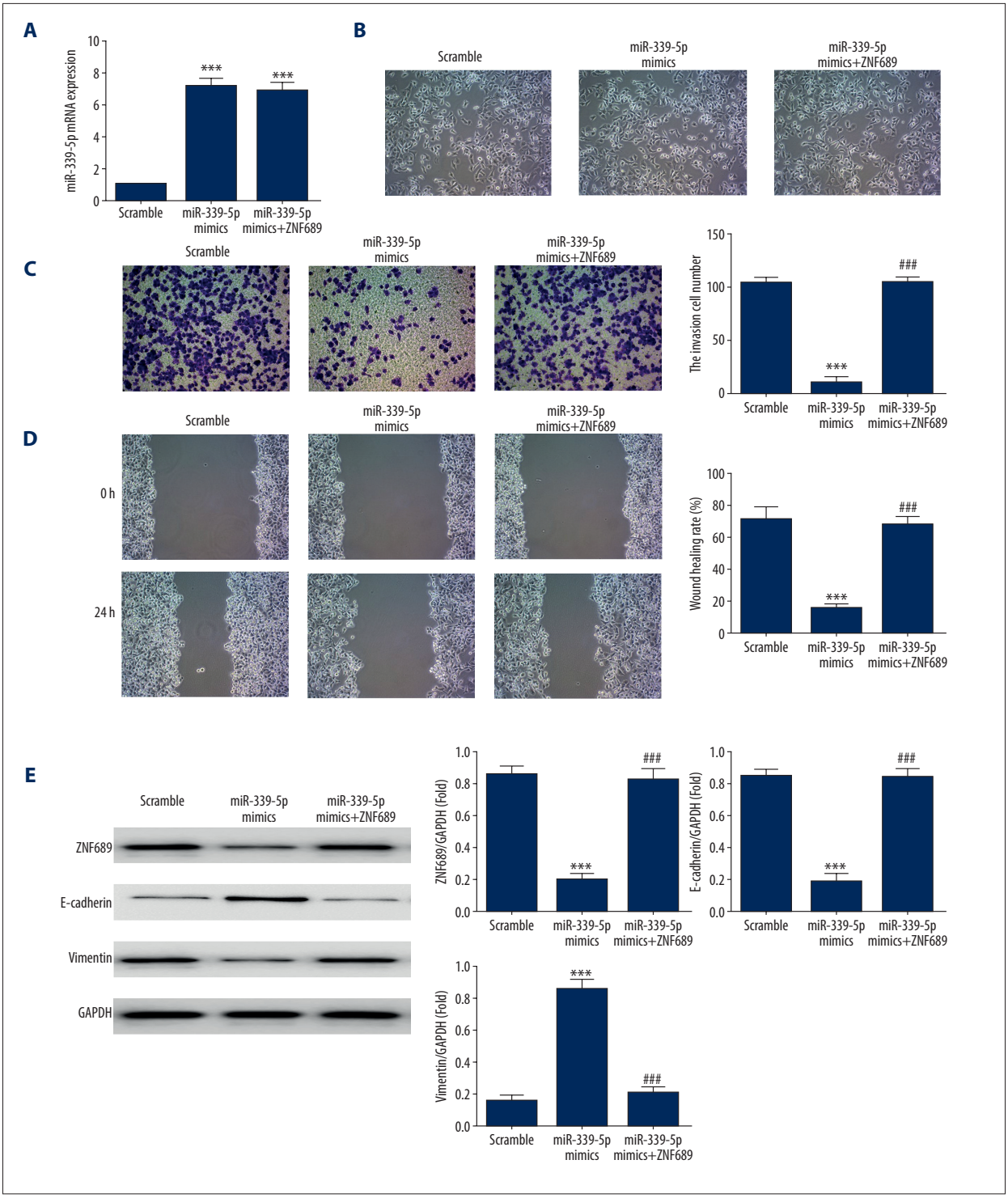

Figure 5. miR-339-5p and ZNF689 had effects to PANC02-H7 cell invasion and migration and relative proteins expression. (**A**) miR-339-5p mRNA expression in difference groups as shown by RT-qPCR. *** P<0.001, compared with scramble group; ### P<0.001, compared with miR-339-5p group. (**B**) The PANC02-H7 cell morphology of different groups. (**C**) The invasion cell number of different groups as shown by Transwell assay. *** P<0.001, compared with scramble group; ### P<0.001, compared with miR-339-5p group. (**D**) The wound healing rate of different groups as shown by wound healing assay. *** P<0.001, compared with scramble group; ### P<0.001, compared with miR-339-5p group. (**E**) The relative proteins expression of difference groups as shown by WB assay. *** P<0.001, compared with scramble group; ### P<0.001, compared with miR-339-5p group.

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miR-339-5p mimics and miR-339-5p inhibitor were transfected into PANC02-H7, and the invasion and migration ability of cells were observed using the Transwell and scratch-wound healing assays. As hypothesized, the low expression of miR-339-5p was observed to be the primary factor associated with increased invasion and migration ability of PANC02-H7 cells.

Previous studies have shown that miR-339-5p is involved in the inhibition of proliferation, migration, and invasion of cancer cells and the regulation of apoptosis-inducing genes. Ren et al. [14] demonstrated that miR-339-5p inhibits the proliferation of lung cancer cells by targeting the 3'-UTR of the Skp2 gene. Shan et al. [15] reported that miR-339-5p inhibits the invasion and migration of ovarian cancer by targeting NACC1 and BCL6. Other studies also reported that miR-339-5p inhibits cell migration and invasion by targeting B cell chronic lymphocytic leukemia/BCL6 [16]. miR-339-5p plays a role as a tumor suppressor in CRC cells via down-regulation of PRL-1 expression [17]. A cancer-based study by Jansson [18] showed that miR-339-5p promotes the function of p53 in MCF-7, CRC and HCT-116 cells by directly targeting the 3'-UTR of MDM2 mRNA to downregulate the expression of MDM2.

TargetScan was used to predict the target of miR-339-5p in the present study, which showed that miR-339-5p directly targeted the 3'-UTR of ZNF689. ZNF689, a member of ZNF protein family, is assumed to be a transcriptional regulator. A previous study reported that the proliferation of cancer cells is effectively inhibited after ZNF689 knockdown [19]. It has also been reported that ZNF689 inhibits apoptosis of hepatocellular carcinoma cell lines by reducing the expression of pro-apoptotic factors [20].

Conclusions

In summary, the overexpression of ZNF689 counteracted the effect of miR-339-5p on the invasion and migration of pancreatic cancer cells. This provides an experimental basis for supporting the potential of miR-339-5p in treating pancreatic cancer by inhibiting ZNF689. Results of the present study also show that the function of miR-339-5p can be attributed to the inhibition of tumorigenesis, primarily by targeting various oncoproteins. This makes miR-339-5p a potential therapeutic target for treating pancreatic cancer.

Conflicts of interest

None.

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