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ORIGINAL ARTICLE

LINC00339 promotes cell proliferation and metastasis in pancreatic cancer via miR-497-5p/IGF1R axis

Renchun Zhang[1]*, Shuqing Hao[2]*, Lei Yang[1], Jiemei Xie[1], Shulin Chen[1], Guiqin Gu[1]

[1]Department of Endocrinology, the Affiliated Yantai Yuhuangding Hospital of Qingdao University, Yantai, China; [2]Department of Hepatology, Yantai City Hospital for Infectious Diseases, Yantai, China.

*These authors contributed equally to this work

Summary

Purpose: *To investigate the role and mechanism of long non-coding (lnc) RNA LINC00339 in pancreatic cancer (PANC), and provide a potential target for its biological diagnosis and treatment.*

Methods: *Quantitative real-time polymerase chain reaction (qRT-PCR) was used to detect the expression of LINC00339 in PANC tissue specimens and cell lines. The experimental cell lines differentially expressing LINC00339 were constructed by using small interfering RNA and lentivirus transfection. Cell proliferation was examined by cell counting kit-8 (CCK-8) and colony formation experiments and transwell experiments were used to assess cell invasion and migration abilities. The luciferase assay and RNA immunoprecipitation (RIP) were employed to study the target gene for LINC00339, and western blot analysis was utilized to measure protein expression of the downstream gene.*

Results: *The level of LINC00339 expression in PANC tissues or cells was significantly higher than that in their respective control groups. Interfering expression of LINC00339 could notably inhibit the proliferation, invasion and migration of SW1990 cells, while the over-expressing expression of LINC00339 obviously increased the growth and metastasis abilities of PANC-1 cells. LINC00339 could act as a miR-497-5p sponge, adsorbing miR-497-5p, thereby inhibiting its action by increasing the expression of its target gene IGF1R. The expression of miR-497-5p and its target gene IGF1R could be significantly altered by altering the expression of LINC00339.*

Conclusions: *LINC00339 was markedly over-expressed in PANC tissues and cells and promoted cell proliferation, invasion, and migration via sponging miR-497-5p, thereby increasing IGF1R expression. Our study could provide a novel target for PANC diagnosis and biotherapy.*

Key words: *IGF1R, LINC00339, metastasis, miR-497-5p, PANC, proliferation*

Introduction

As one of the common malignant tumors in the digestive system, pancreatic cancer (PANC) has a high morbidity and mortality worldwide [1]. According to cancer data from the United States in 2018, the mortality rate of PANC ranks fourth in the entire malignant tumors [2]. Due to its high grade of malignancy, most patients are already in advanced stage when diagnosed and have lost the best period of treatment. Since then, its 5-year survival rate is still less than 5% [3]. Molecular biology research on PANC developed rapidly but its usefulness is still uncertain. Therefore, studying the mechanism of its occurrence and progression is of great significance for the diagnosis and treatment of this disease.

Genomics studies revealed that only 2% of the genes in the human genome have protein-coding functions, and the remaining 98% do not have

Correspondence to: Guiqin Gu, BM. Department of Endocrinology, the Affiliated Yantai Yuhuangding Hospital of Qingdao University, No. 20, Yuhuangding East Rd, Zhifu District, Yantai, 264000 Shandong, China.
Tel: +86 0535-6691999, E-mail: ggq6691999@126.com
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protein-coding effects [4]. Among these, long non-coding RNAs (lncRNAs), which are more than 200 nucleotides in length, are a class of RNA molecules that do not have the ability of encoding proteins [5]. Previously, lncRNAs were considered as 'transcriptional noisy' without any regulating functions. Recently, with a series of studies, lncRNAs were found to be involved in the regulation of various biological functions and mechanisms including tumorigenesis [6]. Tens of thousands of lncRNAs have been found to regulate the proliferation, metastasis, drug resistance and autophagy of tumor cells. For example, lncRNA UPAT promoted colon cancer development via inhibiting UHRF1 degradation and lncRNA HOTAIR accelerated metastasis of esophageal squamous cell carcinoma and indicated a poor prognosis [7,8]. LncRNA PCGEM1 could affect RhoA pathway, inducing tumorigenesis and progression of ovarian carcinoma [9]. Meanwhile, up-regulation of lncRNA NNT-AS1 promoted cell growth and invasion in osteosarcoma while over-expression of lncRNA FAL1 increased proliferation and epithelial-mesenchymal transition (EMT) of non-small cell lung cancer (NSCLC) [10,11]. In contrast, lncRNA OCC-1 suppressed cell proliferation in colorectal cancer by destabilizing HuR protein [12]. In addition, LINC01121 could inhibit cell apoptosis and increase growth and metastasis though inhibiting the Camp/PKA Signaling axis via GLP1R [13]. In PANC, many lncRNAs have also been shown to be differentially expressed and could regulate many biological behaviors of PANC. For example, LINC01133, lncRNA NORAD, and lncRNA LOC389641 could function as tumor-promoter in PANC and accelerate tumorgenesis [14,15]. However, LINC01060 and lncRNA XLOC_000647 suppressed the progression of PANC [16,17].

LINC00339 was a recently discovered oncogenic lncRNA that promoted the development and progression of a variety of tumors. For example, in NSCLC LINC00339 could promote cell proliferation and invasion, while inhibiting cell apoptosis, via affecting FOXM1 expression [18]. In gliomas, LINC00339 expression level was increased and was associated with tumor angiogenesis [19]. However, the expression and role of LINC00339 in PANC have not been studied. In the present study, we found that LINC00339 expression was increased in PANC tissues and cells, therefore, we hypothesized that LINC00339 could play as a cancer-promoting lncRNA in PANC. Furthermore, we investigated that the mechanism of LINC00339 in PANC and found LINC00339 might inhibit the miR-497-5p/IGFR axis to act its oncogene role.

Methods

Tissue samples

A total of 39 PANC tissue samples were from patients who had undergone surgical resection after diagnosis of PANC and 19 control samples were from patients with chronic pancreatitis. Informed consent was signed by each patient. This experiment was conducted with the approval of the Ethics Committee of the Affiliated Yantai Yuhuangding Hospital of Qingdao University. All the tissue specimens were stored in liquid nitrogen immediately after removal.

Cells

Four PANC cell lines (PANC-1, PK-9, BxPC-3, SW1990) and a human normal pancreatic ductal epithelial cell line (HPDE6-C7) were used herein, and were purchased from Type Culture Collection of the Chinese Academy of Sciences (Shanghai, China). The cells were maintained in L15 or RPMI-1640 (Invitrogen, Carlsbad, CA, USA) medium mixed with 10% fetal bovine serum (FBS, Gibco, Rockville, MD, USA).

Cell transfection

Interference of LINC00339 was performed using siRNA sequences synthesized by Genepharma (Shanghai, China). Over-expression of LINC00339 was accomplished using lentiviral transfection. Cells were plated in 6-well plates at a confluence of 50-60%, and equivalent amounts of siRNA or lentiviral fluid were added according to the instructions. Lipofectamine 3000 (Invitrogen, Carlsbad, CA, USA) or polybrene (Genepharma, Shanghai, China) were used to enhance transfection efficiency. Interference sequence (miR-497-5p mimics, inhibitors) of miR-497-5p and its control was synthesized by Genewiz (Suchou, China). Transfection was performed using lipofectamine 3000 according to the instructions. Then, the efficiency of transfection was determined by qRT-PCR.

RNA extraction and quantitative real-time polymerase chain reaction (qRT-PCR)

Total RNA extraction of PANC tissues and cells was performed using TRIZol reagent (Invitrogen, Carlsbad, CA, USA). Reverse transcription was achieved using the PrimeScript RT Reagent Kit (TaKaRa, Tokyo, Japan). The expression levels of LINC00339 and miR-497-5p were determined using SYBR Green I reagent from TaKaRa (Tokyo, Japan) by using ABI 7500 (Foster City, CA, USA). GAPDH (glyceraldheyde 3-phosphate dehydrogenase) was used as an internal reference for LINC00339, and U6 was used as an internal reference for miR-497-5p. Relative expression levels of LINC00339 and miR-497-5p were calculated using the $2^{-\Delta\Delta Ct}$ method.

CCK8 assay

The established cells were processed into single-cell suspension and planted in 96-well plates. Normally placed in the culture medium for 24, 48, 72, 96 hrs, a total of 10 μL CCK8 reagent (TaKaRa, Tokyo,

Japan) was added to each well. Thereafter, after incubating for 2 hrs in the dark, the absorbance at 470 nm per well was measured using spectrophotometer. Each group of experiment was repeated for three times.

Colony formation assay

The treated cells were pipetted into single-cell suspension and planted in 6-well plates. After the culture was normally placed in the medium for 3 weeks, the medium was discarded. Thereafter, the cloned colonies were fixed by adding ice-cold methanol, and then stained with crystal violet, and the number of cell colonies having more than 40 cells was counted.

Transwell assay

Transwell assay was used to assess the ability of cell invasion and migration. For invasion, the transwell inserts were coated with matrigel (BD Biosciences, San Jose, CA, USA). Then, established cells were processed into single-cell suspensions in FBS free medium and planted into the top chamber, while the low chamber was immersed in 10% FBS medium. After 48 -h culture, the inserts were fixed using ice-cold methanol. Cells on the top chamber were removed using cotton swabs and cells on the lower surface were stained with crystal violet. The cells were counted in 6 random fields. For migration assay, the inserts were covered without matrigel and the other steps were the same with the invasion assay.

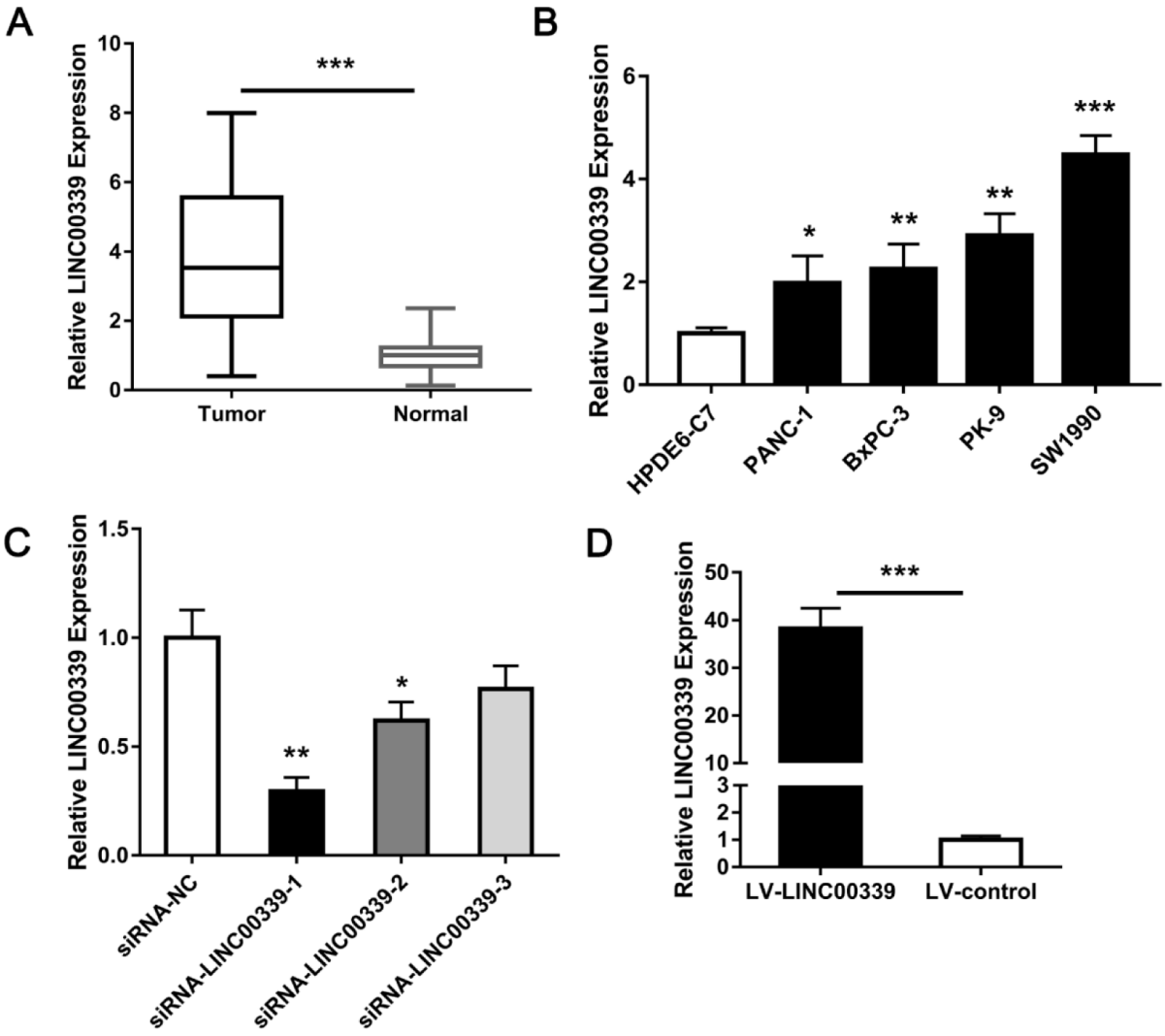

Figure 1. LncRNA LINC00339 was highly expressed in pancreatic cancer (PANC) tissues and cells. **(A):** QRT-PCR shows the lncRNA LINC00339 expression level in 39 PANC tissues and 19 non-tumor tissues. **(B):** LINC00339 expression level in pancreatic cancer cell lines (PANC-1, BxPC-3, PK-9, SW1990) and human normal pancreatic ductal epithelial cell line (HPDE6-C7) showing that the level of LINC0039 in 4 PANC-derived cell lines was significantly higher than that in HPDE6-C7 cells. **(C):** Oligonucleotides targeting LINC00339 (siRNA-LINC00339) and negative controls (siRNA-NC) were transfected into SW1990 cells. The Figure shows that the relative LINC00339 expression in SW1990 cells transfected with siRNA-LINC00339-1 was decreased most. **(D)**: LV-LINC00339 and LV-control were transfected into PANC-1 cells. The Figure shows that overexpression of LINC00339 significantly upregulated LINCC00339 expression in PANC-1 cells comparing with the control group. *p<0.05, **p<0.01, ***p<0.001, compared to control group.

Luciferase reporter assay

Plasmid sequences carrying the mutant or wild type 3'-UTR of LINC00339 or IGF1R were co-treated with the miR-497-5p mimics or control according to the manufacturer's protocol using Lipofectamine 3000. After 48-h culturing, cells were lysed. Dual Luciferase Reporter Assay System (Promega, Madison, WI, USA) was utilized to detect the activity of Renilla luciferase.

RNA immunoprecipitation (RIP)

RNA-Binding Protein Immunoprecipitation Kit (Millipore, Billerica, MA, USA) was purchased for the RIP assays. The binding relationship of LINC00339 and miR-497-5p was analyzed according to the manufacturer's instructions. Experimental cells were lysed and co-treated with buffer containing RIP buffer with human argonaute 2 (Ago2) antibody coated magnetic beads (Millipore, Billerica, MA, USA). For negative control IgG (Millipore, Billerica, MA, USA) was used (input group). RNA was measured using RT-PCR after purified from RNA proteins (TaKaRa, Tokyo, Japan).

Western blot

Cellular protein was extracted using RIPA (radioimmunoprecipitation assay) reagent (Beyotime, Shanghai, China) containing protease inhibitors. After the extraction, the concentration of protein was detected using a BCA (bicinchoninic acid) kit (Beyotime, Shanghai, China). After adding the loading buffer and boiling for 5 min, we used a 10% SDS (sodium dodecyl sulphate) gel for protein electrophoresis. Next, the protein was transferred to a methanol activated PVDF (polyvinylidene fluoride) membrane (Millipore, Billerica, MA, USA). Then, the PVDF membrane was placed in 5% skim milk powder for blocking non-specific sites. After washed

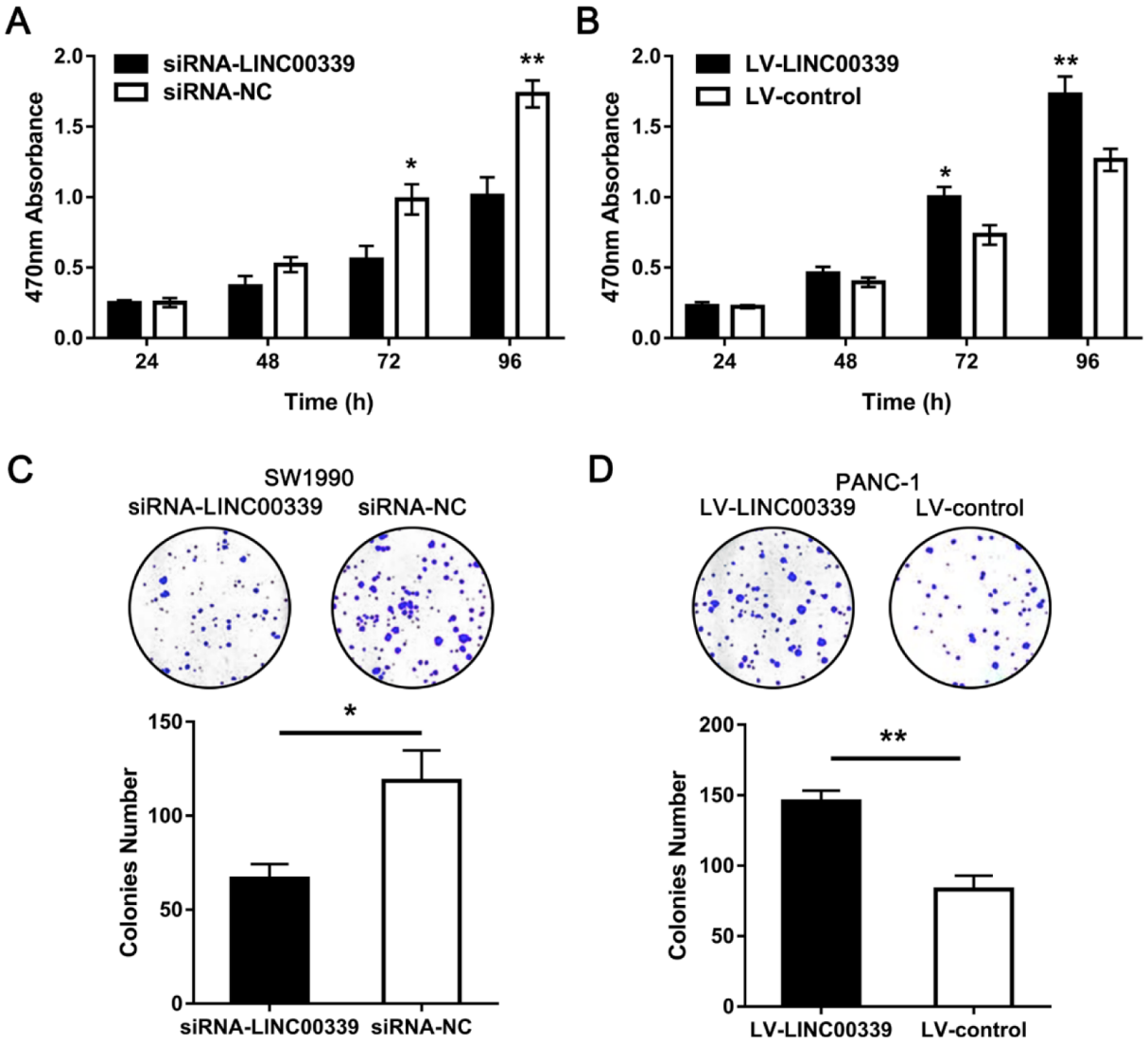

Figure 2. LINC00339 affected the proliferation of PANC cells *in vitro*. **(A,B):** CCK-8 assay shows the proliferation ability of SW1990 cells transfected with siRNA-LINC00339 and siRNA-NC or PANC-1 cells trasfected with LV-LINC00339 and LV-control. **(C,D):** Clone formation assay shows the proliferation ability of SW1990 cells transfected with siRNA-LINC00339 and siRNA-NC or PANC-1 cells trasfected with LV-LINC00339 and LV-control. *p<0.05, **p<0.01, compared to control group.

using tris buffered saline-tween (TBST) for three times, the membrane was incubated in IGF1R and GAPDH primary antibodies (1:1000, Abcam, Cambridge, MA, USA) overnight at 4°C. Afterwards, we immersed the membrane with a horseradish peroxidase-labeled rabbit secondary antibody for 2 hrs. The ECL (electrochemiluminescence) kit (Millipore, Billerica, MA, USA) was used to detect the relative expression of proteins.

Statistics

SPSS (Armonk, IBM, NY, USA) and GraphPad Prism software (La Jolla, CA, USA) were employed to perform the statistical analyses. Student's t-test and one-way ANOVA were employed to analyze the difference and $p<0.05$ was considered as having significance. All these results were shown as mean ± standard deviation (SD).

Results

LINC00339 was over-expressed in PANC tissues and cell lines

To detect the role of LINC00339 in PANC, we detected the expression level of LINC00339 in 39

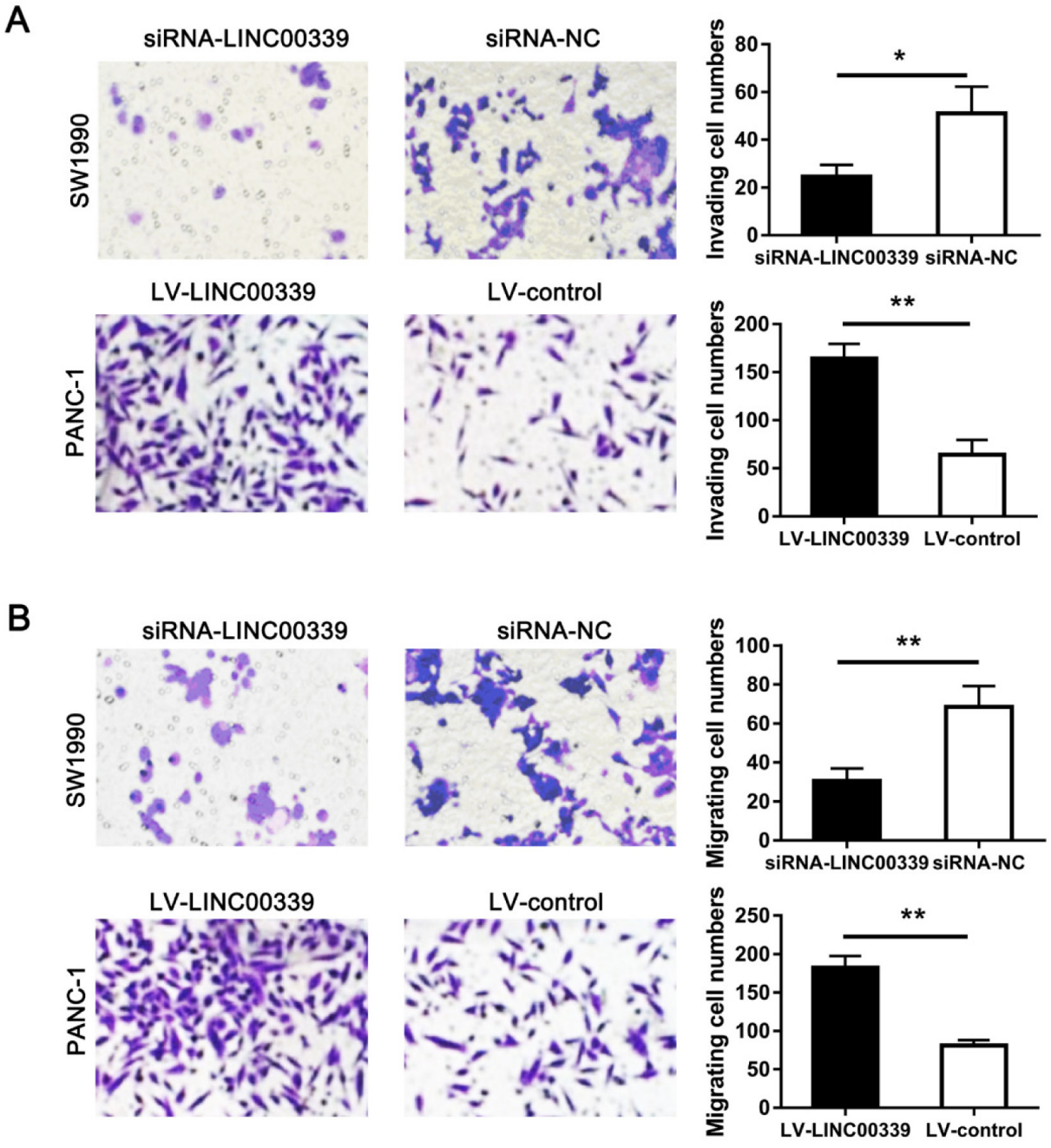

Figure 3. LINC00339 effected the invasion and migration of PANC cells. **(A):** Transwell invasion assay indicates the invaded cell number in established SW1990 cells and PANC-1 cells. **(B):** Transwell migration assay shows the migrated cell number in established SW1990 cells and PANC-1 cells. *p<0.05, **p<0.01, compared to control group.

PANC tissue samples comparing with 19 normal pancreatic tissues using qRT-PCR and found a significantly increased expression of LINC00339 in PANC tissues (Figure 1A). Also, we detected LINC00339 expression in PANC cell lines and found the level of LINC00339 in 4 PANC-derived cell lines was significantly higher than that in the control human normal pancreatic ductal epithelial cell line HPDE6-C7 (Figure 1B). These data suggested that LINC00339 might act as an oncogene in PANC.

To further assess the function of LINC00339 in PANC, we interfered the expression of LINC00339 in SW1990 cells using siRNA-LINC00339-1, siRNA-LINC00339-2, and siRNA-LINC00339-3. Due to the efficiency of knockdown, we chose siRNA-LINC00339-1 as the LINC00339 down-regulation group (Figure 1C). Over-expression of LINC00339 using lentivirus significantly up-regulated LINC00339 expression in PANC-1 cells comparing to the control group (Figure 1D).

Ectopic expression of LINC00339 affected cell proliferation of PANC

To evaluate the influence of LINC00339 in PANC cell proliferation, we employed CCK8 and colony formation assays. As shown in Figure 2A, 2B, knockdown of LINC00339 inhibited cell growth of SW1990 cells, but over-expression of LINC00339 promoted cell proliferation of PANC-1 cells, comparing to each control group. Similarly, SW1990 cells formed less number of colonies than the control group after siRNA-LINC00339 treatment. However, PANC-1 cells presented more colonies

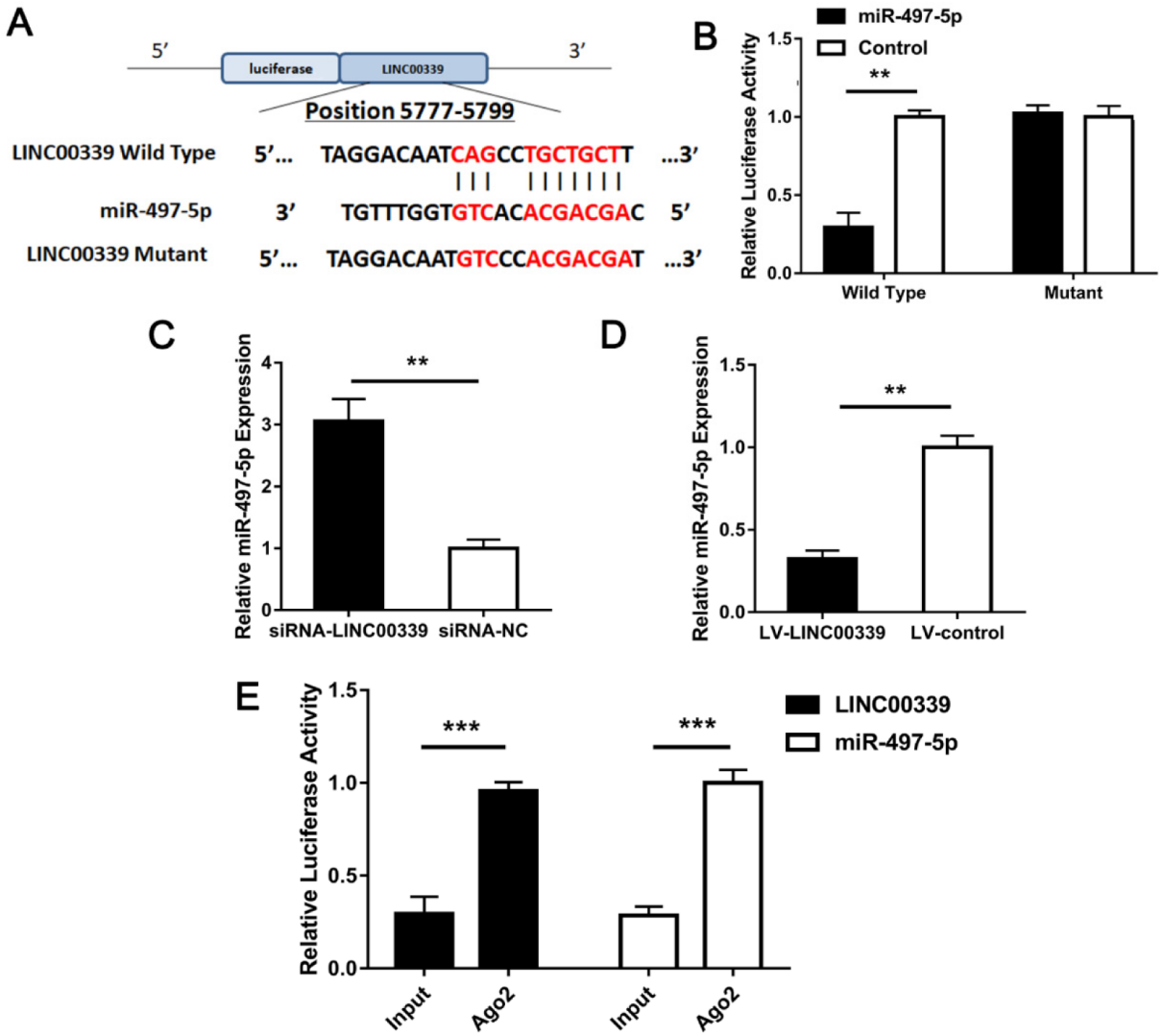

Figure 4. LINC00339 functioned as a sponge of miR-497-5p. **(A):** The sequences of LINC00339 with miR-497-5p, including wild and mutant type. **(B):** Luciferase reporter assay verified the molecular bound within LINC00339 and miR-497-5p. **(C,D):** QRT-PCR shows the miR-497-5p expression level in SW1990 or PANC-1 cells transfected with siRNA-LINC00339 or LV-LINC00339. **(E):** In RIP assay PCR shows the enrichment of LINC00339 and miR-497-5p in Ago2 immunoprecipitate and IgG-pellet. **p<0.01, ***p<0.001, compared to control group.

after LV-LINC00339 transfection (Figure 2C,2D). These data indicated that LINC00339 could promote cell proliferation of PANC cells.

LINC00339 regulated cell invasion and migration in PANC cells

Next, we detected the effect of LINC00339 on cell invasion and migration using transwell assay. Figure 3A showed that down-regulation of LINC00339 markedly decreased cell invasion ability of SW1990 cells but up-regulation of LINC00339 promoted cell invasion of PANC-1 cells comparing with the relative control group. Also, knockdown of LINC00339 inhibited the migrated cell number while over-expression of LINC00339 increased the migrated cell number compared to their negative control (Figure 3B). This assay indicated LINC00339 promoted cell invasion and migration of PANC cells.

LINC00339 acted as a sponge for miR-497-5p

As we identified that LINC00339 could promote cell proliferation, invasion and migration, we further studied the underlying mechanism of

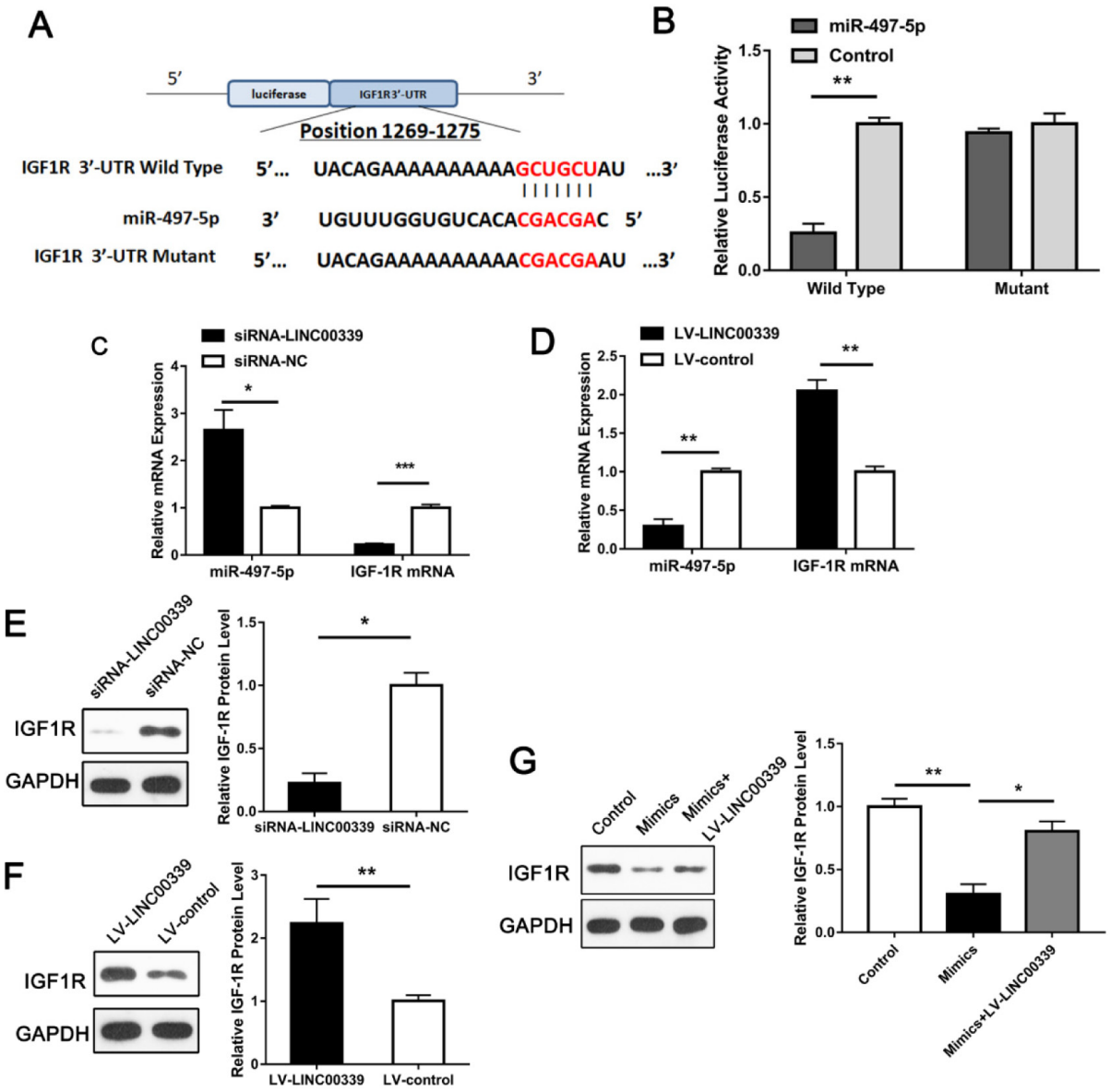

Figure 5. IGF1R was identified as the target protein of miR-497-5p. **(A):** The sequences of IGF1R mRNA 3'-UTR and miR-497-5p, including wild and mutant type. **(B):** Luciferase reporter assay indicates the molecular bound within IGF1R and miR-497-5p. **(C):** QRT-PCR shows the miR-497-5p and IGF1R mRNA expression in SW1990 cells transfected with siRNA-LINC0339 or siRNA-NC. **(D):** QRT-PCR shows the miR-497-5p and IGF1R mRNA expression in PANC-1 cells transfected with LV-LINC00339 or LV-control. **(E,F):** Western blot assay indicates the IGF1R protein expression in established SW1990 cells and PANC-1 cells. **(G):** Western blot assay indicates the IGF1R protein expression in PANC-1 cells transfected with miR-497-5p mimics or LV-LINC00339. *p<0.05, **p<0.01, ***p<0.001 compared to control group.

LINC00339 in PANC. We searched several Bioinformatics databases, and found miR-497-5p could bind to the 3'-UTR of LINC00339 (Figure 4A). Next, we conducted luciferase reporter assay and found that wild type LINC00339 3'-UTR group showed a decrease in luciferase activity but mutant LINC00339 3'-UTR group had no difference, thus indicating the combination between LINC00339 and miR-497-5p (Figure 4B). What's more, miR-497-5p expression level was significantly elevated in SW1990 cells treated with siRNA-LINC00339 compared to siRNA-NC but decreased in PANC-1 cells transfected with LV-LINC00339 compared to LV-control (Figure 4C,4D). Furthermore, RIP assay suggested that LINC00339 and miR-497-5p were remarkably clustered in Ago2 immunoprecipitate when compared to IgG-pellet, indicating miR-497-5p and LINC00339 enriched in the same RNA-induced silencing complex (RISC) (Figure 4E). All these results demonstrated that LINC00339 could sponge miR-497-5p in PANC cells and miR-497-5p was a target for LINC00339.

IGF1R was a direct target for miR-497-5p in PANC

To further detect downstream of LINC00339/miR-497-5p, we next found IGF1R was a potential target for miR-497-5p as there was a binding site in IGF1R 3'-UTR for miR-497-5p (Figure 5A). Also, luciferase assay verified miR-497-5p could bind to the specific region in 3'-UTR of IGF1R (Figure 5B). Moreover, we found miR-497-5p level increased but IGF1R level decreased in siRNA-LINC00339 treated SW1990 cells (Figure 5C). In contrast, miR-497-5p expression was inhibited but IGF1R expression was promoted in LV-LINV00339 trasfected PANC-1 cells (Figure 5D). The expression of IGF1R protein was detected using western blot. Up-regulation of LINC00339 significantly promoted IGF1R protein level while down-regulation of it inhibited IGF1R protein level (Figure 5E, 5F). In addition, we co-trasfected miR-497-5p mimics and LV-LINC00339 in PANC-1 cells and found the expression of IGF1R protein was obviously restored by LV-LINC00339 when compared to the miR-497-5p mimics treated group (Figure 5G). Overall, our study demonstrated that IGF1R was a direct target for LINC00339/miR-497-5p in PANC.

Discussion

The incidence of PANC is on the rise worldwide. According to statistics, the ratio of mortality to morbidity of PANC is 0.99:1. Due to complicated anatomy and pathological features, the therapeutic effect is not satisfactory. The median survival time of patients with PANC is 3 to 5 months, and the 1-year survival rate is less than 10%. The annual increase in the incidence of PANC and its high mortality rate indicate that it is one of the malignant tumors that seriously threaten people's health [1-3]. Therefore, it is important to understand the underlying mechanism of PANC tumorigenesis and progression.

LncRNAs have been a hot topic in recent research on cancer, and participate in the development of many cancers including PANC [5,20]. The role of LINC00339 in promoting proliferation and metastasis of NSCLC and gliomas has also been reported [18,19]. In this study, we first explored the expression of LINC00339 in PANC tissues and found that its expression level was significantly higher compared to normal non-tumor pancreatic tissues. The expression of LINC00339 in PANC-derived cell lines was also significantly elevated. Then, by loss-and gain- of function experiments, we studied the effect of LINC00339 on the proliferation and metastasis of PANC and confirmed that the increase of LINC00339 could significantly promote the proliferation, invasion and migration of PANC cells. However, the interference of LINC00339 greatly reduced the ability of cells to grow and metastasize. These findings verified LINC00339 as an oncogene in PANC as well as in NSCLC or glioma.

LncRNAs could exert their effects by adsorbing miRNAs as a sponge, and this mechanism has been confirmed in many studies. For instance, lncRNA MT1JP suppressed the proliferation and migration of gastric cancer cells via sponging miR-214-3p, and LINC01234 regulated CBFB expression functioning as a sponge for miR-204-5p in gastric cancer. In addition, in glioma, LINC00152 acted as a competing endogenous RNA of miR-16 and increased cell growth and metastasis [21-23]. Therefore, we searched through several databases and found that the 3'-UTR of LINC00339 could provide a site for miR-497-5p binding. MiR-497-5p was reported to inhibit tumorigenesis and progression of several cancers including osteosarcoma, angiosarcoma and hepatocellular carcinoma [24-26]. We confirmed the direct binding relationship between LINC00339 and miR-497-5p through the luciferase assay and RIP assay. At the same time, changes in the expression level of LINC00339 could also cause opposite changes in the level of miR-497-5p. This confirmed that LINC00339 functioned as a miR-497-5p sponge to regulate the level of miR-497-5p and exerted its regulatory effects. Furthermore, we identified IGF1R as a direct target of LINC00339/miR-497-5p in PANC. Insulin-like growth factor-I receptor (IGFIR) could promote cell proliferation and metastasis of cervical cancer, colorectal cancer

and pancreatic ductal adenocarcinoma, and could also function as a biomarker for prostate cancer [26-29]. Here, the binding relationship of miR-497-5p and IGF1R was confirmed and LINC00339 could influence the expression level of IGF1R mRNA and protein. Moreover, LINC00339 over-expression rescued IGF1R protein level decreased by miR-497-5p, which verified the axis of LINC00339/miR-497-5p/IGF1R.

Conclusion

Taken all together, our study demonstrated for the first time the relationship between LINC00339 and PANC, confirming the apparent increase in the expression of LINC00339 in PANC tissues and cells, while LINC00339 promotes proliferation, invasion and migration of PANC cells. Furthermore, we confirmed that LINC00339 could act as a sponge of miR-497-5p, adsorbing miR-497-5p, and then increasing the expression of IGF1R to exert its cancer-promoting effect. These findings might provide a new marker for the diagnosis of PANC while providing a new target for its biotherapeutics.

Conflict of interests

The authors declare no conflict of interests.

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