



Inhibition of TUG1/miRNA-299-3p Axis Represses Pancreatic Cancer Malignant Progression via Suppression of the Notch1 Pathway

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Abstract

Background and Aims Taurine-upregulated gene 1 (TUG1) is reported to be upregulated and contributes to the progression of Pancreatic cancer (PC) by serving as an oncogene. Our aims were to explore the precise mechanism of TUG1 involved in PC pathogenesis.

Methods TUG1 and miR-299-3p expression profiles were measured by qRT-PCR. The direct interaction between TUG1 and miR-299-3p was explored by luciferase reporter assay. MTT assay, flow cytometry analysis, caspase-3 activity assay, Transwell invasion assay and wound healing assay were performed to evaluate cell proliferative ability, apoptosis, caspase-3 activity, invasion and migration, respectively. Western blot was conducted to examine the expressions of Ki67, Bax, Bcl-2, matrix metalloproteinase-2 (MMP-2), MMP-9, E-cadherin, N-cadherin, Snail, Notch1, Survivin, and CyclinD1. In addition, animal experiments were also implemented.

Results TUG1 was highly expressed, while miR-299-3p was underexpressed in PC tissues and PC cells. Furthermore, the significant increase of TUG1 in PC tissues of advanced patients (stage 3/4) was observed compared to patients (stage 1/2). TUG1 was negatively correlated with miR-299-3p expression in PC tissues. Moreover, TUG1 functioned as a molecular sponge of miR-299-3p to repress its expression. TUG1 knockdown suppressed cell proliferation, invasion, migration, and epithelial-mesenchymal transition (EMT), and induced apoptosis in PC cells, and repressed tumor growth and EMT in PC xenograft models, which were reversed following reintroduction with anti-miR-299-3p. Furthermore, we found that TUG1 silencing inactivated the Notch1 pathway in PC by upregulating miR-299-3p.

Conclusions The results reported that inhibition of TUG1/miR-299-3p axis suppressed PC malignant progression via suppression of the Notch1 pathway.

Keywords TUG1 · miR-299-3p · Notch1 pathway · EMT · Pancreatic cancer

Introduction

Pancreatic cancer (PC) is recognized as one of the most aggressive human malignancies and ranks the third leading cause of cancer-associated mortality, with an increasing incidence rate worldwide [1]. In 2018, there were an estimated 55,440 newly diagnosed cases and 44,330 deaths annually due to PC in the USA [2]. Surgical resection remains the most effective curative therapy for PC, but only pancreatic tumors of 15–20% of patients are able to be

removed surgically when diagnosed [3]. Despite considerable advances made in PC oncological treatment regimens in recent years, its prognosis is dismal mainly due to non-specific later symptoms, distant invasion and liver metastasis, with a 5-year overall survival of <5% and a medium overall survival time of approximately 6 months [2]. In this regard, a better understanding of the molecular mechanisms underlying PC progression is desperately needed to explore effective treatment approaches for PC.

Human genome sequence shows that only approximately 2% of human genome can be translated into proteins, whereas the vast majority is noncoding RNAs, which are key regulators in cancer biology as oncogenes or tumor suppressors [4]. Long noncoding RNAs (lncRNAs), a group of noncoding transcripts with more than 200 nucleotides in length, exert important roles in a wide range of biological

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processes, including cell growth, apoptosis, metastasis and tumorigenesis [5]. Dysregulation of lncRNAs is reportedly associated with the pathogenesis and development of numerous cancers, including PC [6]. LncRNA taurine-upregulated gene 1 (TUG1), located at the chromosome 22q12.2, is originally identified as a transcript upregulated in taurine-treated developing mouse retinal cells [7]. It has been shown that TUG1 is frequently deregulated and participates in the development and progression of several types of cancers [8]. Previously, it is reported that TUG1 is upregulated in PC tissues and cells and contributes to the progression of PC by serving as an oncogene [9–11]. However, the precise mechanism of TUG1 involved in PC pathogenesis is still unclear.

MicroRNAs (miRNAs) are a sort of highly conserved small noncoding RNAs (~20 nucleotides in length) that suppress gene expression at the post-transcriptional level by base pairing with the 3'-untranslated regions (3'UTRs) of target mRNA [12]. Increasing evidence has demonstrated the close association between aberrantly expressed miRNAs and various malignant tumors including PC [13]. Increasing studies have proved that lncRNAs serve as competing endogenous RNAs (ceRNAs) for miRNAs via base-pair targeting, thereby counteracting miRNA-mediated repression of the specific targets of miRNA [14]. miR-299-3p, located on chromosome 14q32.31, is frequently dysregulated in many types of cancers [15, 16]. However, whether TUG1 could serve as a molecular sponge of miR-299-3p in PC cells remains to be further explored.

In our study, we found that TUG1 was highly expressed, while miR-299-3p was underexpressed in PC tissues and cells. Functional and mechanistic analyses revealed that TUG1 knockdown inhibited PC malignant progression *in vitro* and *in vivo* by sponging miR-299-3p via suppressing the Notch1 pathway. The TUG1/miR-299-3p/Notch1 pathway may have therapeutic potential for PC carcinogenesis.

Materials and Methods

Clinical Samples

A total of thirty-one PC tissues, matched adjacent non-tumor tissues, and eight normal tissues were acquired from patients who underwent surgery or percutaneous biopsy between September 2016 and September 2018 at the First Affiliated Hospital of Zhengzhou University. There were 15 PC cases at stage 1/2 and 16 PC cases at stage 3/4, which were classified according to the Tumor Node Metastasis (TNM) staging system of the American Joint Committee on Cancer (AJCC) and the International Union against Cancer (UICC). All the enrolled patients did not undergo any adjuvant therapies including radiotherapy or chemotherapy prior to sample collection. All tissue samples were subjected

to immediate freezing after excision and storage in liquid nitrogen until use. Signed informed consent was collected from all patients prior to sample collection, and the study was approved by the Ethics committee of the First Affiliated Hospital of Zhengzhou University.

Cell Culture and Transfection

Human PC cell lines (PANC-1 and BXPC-3), human pancreatic ductal epithelium cells, HPDE and human embryo kidney cell line 293T were gained from Cell Bank (Chinese Academy of Sciences, Shanghai). Human PC cell lines (PSN-1 and HPAC) were purchased from American Type Culture Collection (ATCC, Manassas, VA, USA). PANC-1, BXPC-3 and PSN-1 cells were incubated in Dulbecco's modified Eagle's medium (DMEM; Gibco, Carlsbad, CA, USA) supplemented with 10% heat-inactivated fetal bovine serum (FBS; Gibco), 100 U/mL penicillin, and 100 µg/mL streptomycin and cultured in a humidified CO₂ incubator at 37 °C. HPAC cells were cultured in RPMI1640 (Gibco).

Vector Constructs

Lentiviral short hairpin RNA (shRNA) specifically targeting TUG1 (sh-TUG1) or miR-299-3p (anti-miR-299-3p), miR-299-3p mimics (miR-299-3p) and respective control groups were purchased from GeneCopoeia (Guangzhou, China). sh-TUG1 or anti-miR-299-3p were cloned and ligated into the pLenti-hU6-EF1-GFP-Puro lentivirus plasmid, and the constructs were named as pLenti-sh-TUG1 and pLenti-anti-miR-299-3p, respectively.

Lentivirus Production and Infection

pLenti-sh-TUG1 or pLenti-anti-miR-299-3p plasmid was cotransfected into 293T cells along with the packaging plasmids (pCMV-Δ8.2 and pCMV-VSV-G) according to the previous reported method [17]. After 48 h, the lentiviral particles were harvested by centrifuging at 25,000 rpm for 1.5 h at 4 °C and then infection was performed to gain the PANC-1 and BXPC-3 cells expressing sh-TUG1 or/and anti-miR-299-3p.

RNA Extraction and Quantitative Real-Time PCR (qRT-PCR)

Total RNA was extracted from collected tissues and cultured cells using TRIzol reagent (Invitrogen). Reverse transcription into complementary DNA (cDNA) was performed by PrimeScript RT Reagent kit (Invitrogen). TUG1 and miR-299-3p expressions were detected using SYBR Green Real-Time PCR Master Mix (Invitrogen) and mirVana™ miRNA Detection kit (Invitrogen) on

a StepOnePlus real-time PCR system (Applied Biosystems, Foster City, CA, USA), respectively. GAPDH and U6 small nuclear RNA were used as the normalization. The relative fold changes of target genes were calculated using the $2^{-\Delta\Delta Ct}$ approach.

The primer sequences were as followed: TUG1, forward: 5'-AGGCATGTCTTTGACCCCAGG-3', reverse: 5'-GCTTTACTGGGTGCCATT-3'; miR-299-3p, forward: 5'-ACACTCCAGCTGGGTATGTGGGATGGTAAA C-3', reverse: 5'-CTCAACTGGTGTCTGGAGTCGGC AATT-3'.

3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazoliumbromide (MTT) Assay

Cell proliferative ability was assessed by MTT assay. Briefly, the infected PANC-1 and BXPC-3 cells were plated into 96-well plates. Following 1 day, 2 days and 3 days of incubation, 20 μ L of MTT solution (0.5 mg/mL; Sigma-Aldrich, St Louis, MO, USA) was added into each well and incubated for an additional 4 h at 37 °C. Then, the culture medium was discarded before the addition of 150 μ L dimethyl sulfoxide (DMSO). The absorbance at 490 nm was recorded using a microplate reader (Molecular Devices, Sunnyvale, CA, USA).

Flow Cytometry Analysis

Apoptosis of the cells was detected using the PE-Annexin V/7-ADD Apoptosis Detection Kit (BD Pharmingen, San Diego, CA, USA). Briefly, approximately 2×10^5 cells were harvested and washed twice with ice-cold PBS. Following resuspending in 400 μ L $1 \times$ binding buffer, cells were double stained with PE-Annexin-V and 7-ADD for 15 min at room temperature in the dark. The apoptotic cells were analyzed by a FACScan flow cytometer (Beckman Coulter, Fullerton, CA, USA).

Caspase-3 Activity Assay

Cell apoptosis was also evaluated by measurement of caspase-3 activity. Caspase-3 activity of PANC-1 and BXPC-3 cells was detected using Caspase-3 Colorimetric Activity Assay Kit (Beyotime, Shanghai, China) referring to the manufacturer's guide.

Western Blot Analysis

Total protein was extracted from tissues and cultured cells using RIPA lysis buffer (Solarbio, Beijing, China). Protein was subjected to 10% SDS-PAGE, followed by electrotransfer onto nitrocellulose (NC) membranes (Millipore, Billerica, MA, USA). After being blocked

with 5% non-fat milk in Tris-buffered saline containing 0.25% Tween-20 for 2 h, the membranes were probed with primary antibodies against Ki67 (#SAB4501880; Sigma-Aldrich), Bax (#5023; Cell Signaling Technology, Danvers, MA, USA), Bcl-2 (#2827; Cell Signaling Technology), matrix metalloproteinase-2 (MMP-2) (#87809; Cell Signaling Technology), MMP-9 (#3852; Cell Signaling Technology), E-cadherin (#3199; Cell Signaling Technology), N-cadherin (#4061; Cell Signaling Technology), Snail (#3879; Cell Signaling Technology), Notch1 (#4380; Cell Signaling Technology), Survivin (#2808; Santa Cruz Biotechnology), CyclinD1 (#2922; Cell Signaling Technology), and GAPDH (#2118; Cell Signaling Technology) at 4 °C overnight, followed by incubation with horseradish peroxidase (HRP)-conjugated secondary antibody (#14709; Cell Signaling Technology) for 1 h at room temperature. Finally, protein signals were detected using an enhanced chemiluminescence detection kit (Roche Diagnostics GmbH, Mannheim, Germany).

Luciferase Reporter Assay

The wild-type fragments of TUG1 containing the potential binding sites of miR-299-3p were synthesized and subcloned into pmiR-GLO luciferase reporter plasmids (Promega, Madison, WI, USA) to generate TUG1-WT. The mutant miR-299-3p binding sites within TUG1 luciferase reporter vector (TUG1-MUT) were also constructed. 293T cells were plated into 96-well plates at density of 1×10^4 cells/well and cotransfected with TUG1-WT or TUG1-MUT, together with 2 ng of pRL-TK (Promega) and miR-299-3p or miR-NC using Lipofectamine 3000 (Invitrogen). Firefly and Renilla luciferase activities were detected using a Dual-Luciferase Reporter Assay System (Promega) after 48 h of transfection.

Transwell Invasion Assay

Cell invasion ability was determined using Transwell invasion assay. 2×10^4 PANC-1 cells resuspended in 100 μ L serum-free culture medium were seeded into the upper chambers precoated with Matrigel (BD Biosciences, Franklin, Lakes, NJ, USA) while 500 μ L culture medium supplemented with 10% FBS was added to the lower chambers. After incubation for 24 h, the noninvasive cells on the upper membrane were scraped off with a cotton bud, and the cells that had invaded to the lower membranes were fixed with 4% paraformaldehyde and stained with 0.1% crystal violet. The number of invasive cells was assessed in five fields under an optical microscope (Olympus Crop, Tokyo, Japan) at a magnification of 200 \times .

Wound Healing Assay

Cell migratory ability of BXPC-3 cells was determined by wound healing assay. BXPC-3 cells were seeded into 6-well plates at density of 2×10^5 cells/well. An artificial wound was uniformly made by scratching cell monolayers with a 10 μ L sterile pipette tips when grown to 90% confluence. Cells were washed with PBS to remove the detached cells and then maintained for 24 h in serum-free DMEM medium. Cell migratory ability was evaluated by measuring the wound closure under a microscopy (Olympus, Tokyo, Japan) at a magnification of 40 \times .

Animal Experiments

Five-week-old male BALB/c nude mice were housed under specific pathogen-free conditions. Approximately 1×10^6 PANC-1 cells transfected with sh-NC, sh-TUG1, sh-TUG1 + anti-miR-NC, or sh-TUG1 + anti-miR-299-3p were subcutaneously injected into the left subaxillary of the mice. Tumor volume was routinely recorded every 5 days and calculated according to the formula: Volume = (Length \times Width²)/2. The tumor-bearing mice were killed at day 30 post-inoculation, and tumors were surgically removed and weighed. The resected tumor tissues were fixed in formalin and embedded in paraffin for immunohistochemistry (IHC) assay. All animal experiments were performed with the approval of the Animal Care and Use Committee of the First Affiliated Hospital of Zhengzhou University.

IHC Assay

The sections (4 μ m thickness) from paraffin-embedded blocks were dewaxed with xylene and rehydrated through graded concentration of ethanol. After antigen retrieval, slides were then immersed in 3% hydrogen peroxide for 10 min to block endogenous peroxidases. Subsequently, the sections were blocked with 10% bovine serum albumin (BSA) and incubated with primary antibody against Ki67 at 4 $^{\circ}$ C overnight, followed by incubated with HRP-conjugated secondary antibody for 1 h at room temperature. Finally, the sections were visualized using 3,3'-diaminobenzidine (DAB) and counterstained with hematoxylin. The stained sections were imaged using an optical microscope (Olympus).

Statistical Analysis

All results were shown as mean \pm standard deviation (SD). All statistical analyses were conducted using SPSS version 21.0 software (SPSS Inc., Chicago, IL, USA) with Student's *t* test. Differences were considered statistically significant at $P < 0.05$.

Results

High TUG1 and Low miR-299-3p Expressions in PC Tissues and Cells

Firstly, TUG1 expression in 31 paired PC tissues, adjacent non-tumor and 8 normal tissues was detected. As demonstrated by qRT-PCR analyses, TUG1 expression was significantly upregulated in PC tissues compared with that in adjacent non-tumor and normal tissues (Fig. 1a). Moreover, we found that TUG1 expression was much higher in PC patients at stage 3/4 than that at stage 1/2 (Fig. 1b). As shown in Table 1, there exists significant differences in the distribution of lymphatic metastasis ($X^2 = 9.314$, $P = 0.002$) and TNM stage ($X^2 = 23.934$, $P = 0$) between high expression of TUG1 group and low expression of TUG1 group, which showed that high expression level of TUG1 was closely associated with lymphatic metastasis and advanced stage. Additionally, TUG1 expression was also increased in PC cell lines (PSN-1, PANC-1, HPAC, and BXPC-3) relative to that in HPDE cells (Fig. 1c). miR-299-3p expression in PC tissues was detected by qRT-PCR. As displayed in Fig. 1d, miR-299-3p expression was aberrantly downregulated in PC tissues relative to that in adjacent non-tumor and normal tissues. Also, expression of miR-299-3p was much lower in PC cells BXPC-3 and PANC-1 than that in HPDE cells (Fig. 1e). Pearson's correlation analysis demonstrated that TUG1 was negatively correlated with miR-299-3p expression in PC tissues (Fig. 1f). These results suggested that TUG1 and miR-299-3p were aberrantly expressed in PC tissues and cells and might play crucial roles in pancreatic cancer malignant progression.

TUG1 Functioned as a ceRNA of miR-299-3p to Negatively Regulate Its Expression

Through online bioinformatics analysis via StarBase software, the potential binding sites of miR-299-3p in TUG1 were predicted (Fig. 2a). Furthermore, luciferase reporter assay implicated that miR-299-3p overexpression dramatically reduced the luciferase activity of TUG1-WT, but failed to affect the luciferase activity of TUG1-MUT in 293T cells (Fig. 2b). In addition, knockdown of TUG1 significantly enhanced miR-299-3p expression in PANC-1 and BXPC-3 cells (Fig. 2c–f). These results revealed that TUG1 acted as a ceRNA for miR-299-3p to repress its expression in PC cells.

TUG1 Knockdown Repressed Cell Proliferation and Induced Apoptosis in PC Cells by Upregulating miR-299-3p

To investigate the effects of TUG1 or along with miR-299-3p on PC cell biological behaviors, functional experiments were

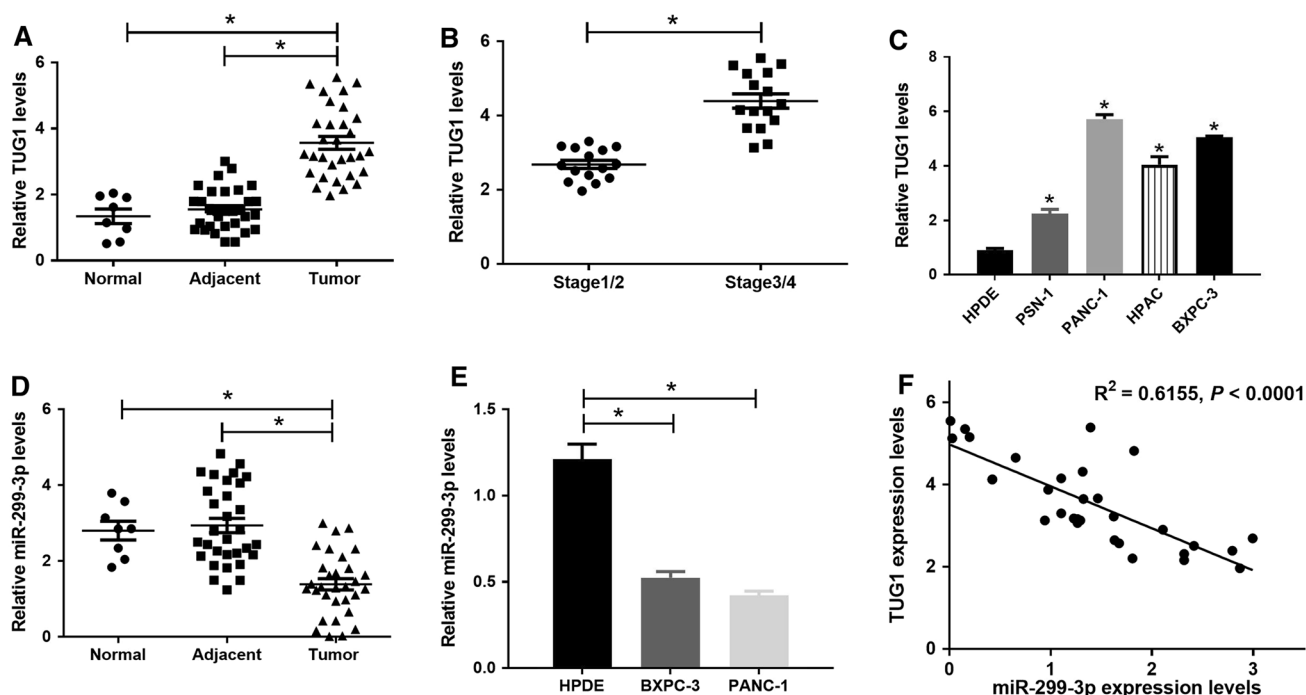


Fig. 1 The expression profiles of TUG1 and miR-299-3p in PC tissues and cells. **a, d** The expressions of TUG1 and miR-299-3p in 31 paired PC tissues, adjacent non-tumor tissues and 8 normal tissues were detected by qRT-PCR. **b** The expression of TUG1 in 15 PC patients with TNM stage 1/2 and 16 PC patients with TNM stage 3/4 was examined by qRT-PCR. **c** TUG1 expression in PC cell lines

(PSN-1, PANC-1, HPAC, and BXPC-3) and human pancreatic ductal epithelium cells HPDE was determined by qRT-PCR. **e** qRT-PCR analysis of miR-299-3p expression in PC cells (BXPC-3 and PANC-1) and HPDE cells. **f** The correlation between TUG1 and miR-299-3p in PC tissues. * $P < 0.05$

Table 1 Pancreatic cancer patient characteristics and TUG1 expression in tumor tissues

Variables	Patients (n)	High expression of TUG1 (n)	Low expression of TUG1 (n)	X^2	P value
Sex					
Male	19	10	9	0.02	0.886
Female	12	6	6		
Age					
≤ 60	11	5	6	0.259	0.611
> 60	20	11	9		
Tumor location					
Head and neck of pancreas	22	9	13	3.476	0.062
Body and tail of pancreas	9	7	2		
Tumor size (cm)					
≤ 5	28	14	14	0.301	0.583
> 5	3	2	1		
Lymphatic metastasis					
Yes	17	13	4	9.314	0.002
No	14	3	11		
TNM stage					
I+II	15	0	15	23.934	0
III+IV	16	14	2		

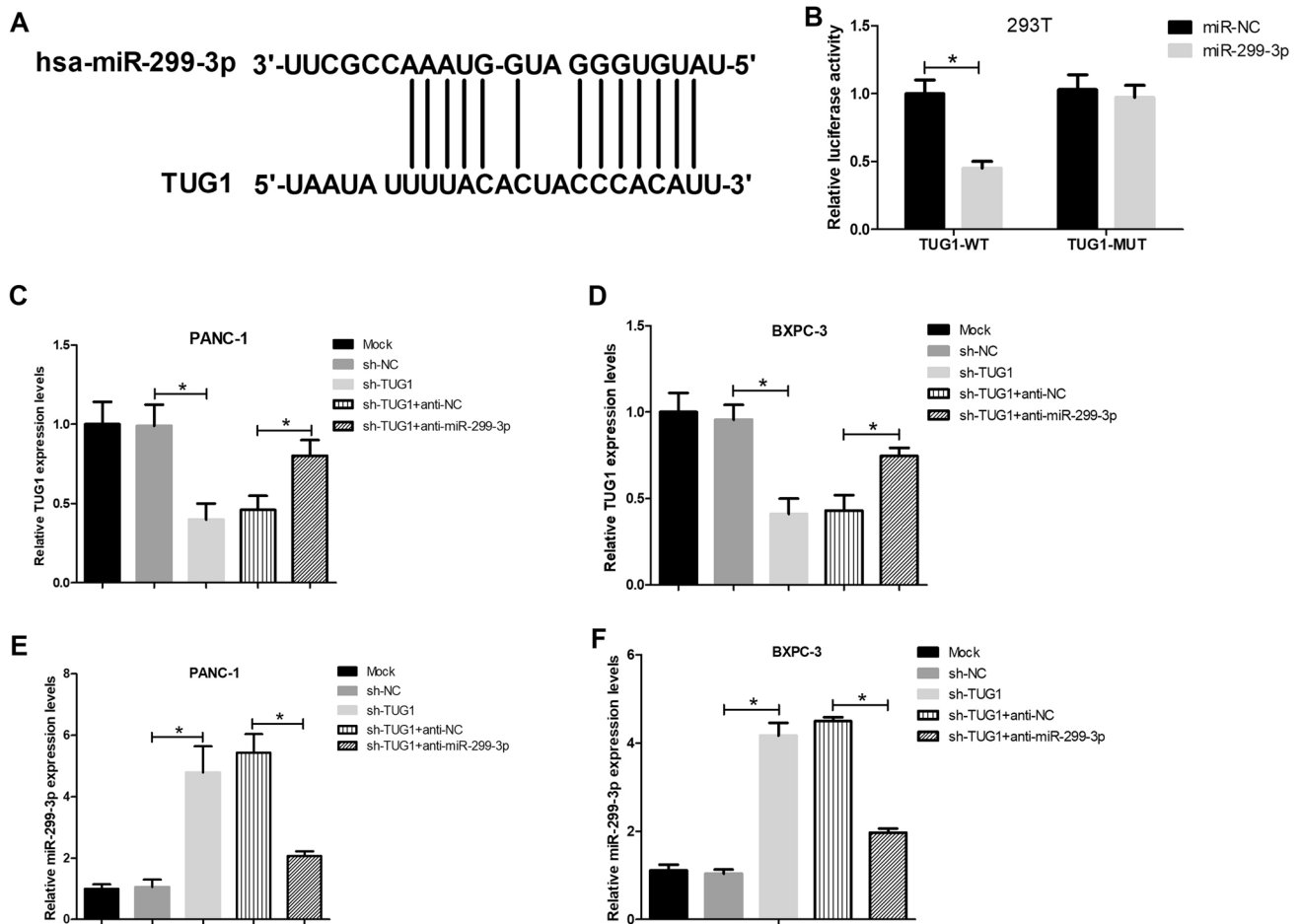


Fig. 2 The interaction between TUG1 and miR-299-3p. **a** Sequence alignment of miR-299-3p with the predicted binding sites in the wild-type region of TUG1. **b** The interaction between TUG1 and miR-299-3p was verified by luciferase reporter assay. **c-f** The expression

of TUG1 and miR-299-3p in PANC-1 and BXPC-3 cells transfected with sh-TUG1, sh-NC, or combined with anti-miR-299-3p or anti-NC was detected by qRT-PCR. * $P < 0.05$

performed. MTT assay showed that delivery of sh-TUG1 greatly impeded the proliferation of PANC-1 and BXPC-3 cells relative to sh-NC group, which was remarkably ameliorated following reintroduction with anti-miR-299-3p (Fig. 3a). Consistently, western blot proved that the expression of Ki67, a marker of cell proliferation, was dramatically inhibited by TUG1 silencing in PANC-1 and BXPC-3 cells, whereas this effect was markedly restored by miR-299-3p inhibition (Fig. 3b). In addition, flow cytometry analysis hinted that the percentage of apoptotic PANC-1 and BXPC-3 cells was notably increased in sh-TUG1 group, which was conspicuously reversed in sh-TUG1 + anti-miR-299-3p group (Fig. 3c). Moreover, TUG1 depletion potently elevated Bax level and reduced Bcl-2 level in PANC-1 and BXPC-3 cells versus control group, which were prominently reversed in response to inhibition of miR-299-3p (Fig. 3d). Furthermore, caspase-3 activity assay presented that sh-TUG1-transfected PANC-1 and BXPC-3 cells showed a

notable enhancement of caspase-3 activity compared to sh-NC-introduced group, while miR-299-3p suppression strikingly antagonized the facilitative effect of TUG1 depletion on caspase-3 activity (Fig. 3e). Collectively, these results demonstrated that TUG1 knockdown repressed cell proliferation and induced apoptosis in PC cells by upregulating miR-299-3p.

TUG1 Knockdown Inhibited Cell Invasion and Migration in PC Cells by Upregulating miR-299-3p

Transwell invasion assay showed that depletion of TUG1 substantially restrained cell invasive abilities in PANC-1 cells, while miR-299-3p inhibition remarkably abolished the inhibitory influence of TUG1 silencing on cell invasive ability (Fig. 4a). Meanwhile, we found that TUG1 knockdown caused a significant decrease in wound closure when compared with control group according to wound healing assay,

which was notably recovered following reintroduction with anti-miR-299-3p (Fig. 4b). In addition, western blot analysis demonstrated that TUG1 silencing apparently hindered that the expressions of MMP-2 and MMP-9 in PANC-1 and BXPC-3 cells with respect to control group, while anti-miR-299-3p drastically overturned the effects of TUG1 silencing on MMP-2 and MMP-9 expressions (Fig. 4c). Therefore, we concluded that TUG1 silencing inhibited cell invasion and migration in PC cells by upregulating miR-299-3p.

TUG1 Silencing Retarded Epithelial-Mesenchymal Transition (EMT) Progress and the Notch1 Pathway in PC Cells by Upregulating miR-299-3p

Compared with sh-NC group, we demonstrated that E-cadherin expression was noticeably enhanced and N-cadherin and Snail expressions were significantly reduced in sh-TUG1-transfected PANC-1 and BXPC-3 cells (Fig. 5a, b). However, anti-miR-299-3p distinctly reversed the effects of TUG1 on E-cadherin, N-cadherin and Snail in PANC-1 and BXPC-3 cells (Fig. 5a, b), suggesting that TUG1 silencing retarded EMT process in PC cells by upregulating miR-299-3p. Recent studies have demonstrated that TUG1 is identified as a Notch downstream target in glioma [18]. To clarify the mechanism underlying the oncogenic role of TUG1 in PC cells, we explored the effects of sh-TUG1 or along with anti-miR-299-3p on the Notch1 pathway in PC cells. As illustrated by western blot, the expressions of Notch1, Survivin, and CyclinD1 were considerably curbed in response to TUG1 knockdown in PANC-1 and BXPC-3 cells, which were obviously relieved after inhibition of miR-299-3p (Fig. 5c, d), indicating that TUG1 knockdown inactivated the Notch1 pathway in PANC-1 and BXPC-3 cells by upregulating miR-299-3p.

TUG1 Knockdown Suppressed PC Xenograft Growth and EMT by Upregulating miR-299-3p via Inactivating the Notch1 Pathway

To reveal the effect of TUG1 or combined with miR-299-3p on the progress of PC in vivo, the xenograft tumor model using PANC-1 cells that were transfected with sh-TUG1, sh-NC, or combined with anti-miR-299-3p or anti-NC was established. As shown in Fig. 6a, b, TUG1 depletion dramatically inhibited tumor growth in comparison with control group, while these effects were effectively recuperated by anti-miR-299-3p. IHC assay demonstrated that Ki67 expression was significantly decreased in sh-TUG1 group relative to sh-NC group, but strikingly restored in sh-TUG1 + anti-miR-299-3p group (Fig. 6d). In line with the in vitro results, the expressions of E-cadherin was increased, and N-cadherin and Snail expressions were decreased in sh-TUG1-transfected group relative to sh-NC group, whereas these effects

were reversed in sh-TUG1 + anti-miR-299-3p-introduced group (Fig. 6e). Moreover, tumors formed from sh-TUG1-transfected PANC-1 cells exhibited a significant decline in the protein expressions of Notch1, Survivin, and CyclinD1 in comparison with that formed from sh-NC-treated PANC-1 cells, which was greatly ameliorated in tumors formed from sh-TUG1 + anti-miR-299-3p-introduced PANC-1 cells (Fig. 6f). Additionally, miR-299-3p expression was remarkably increased in tumor tissues from sh-TUG1 group relative to that in sh-NC group, which was apparently attenuated in tumor tissues from sh-TUG1 + anti-miR-299-3p group (Fig. 6c). These results suggested that TUG1 knockdown suppressed PC malignant progress in vivo by upregulating miR-299-3p via inactivating the Notch1 pathway.

Discussion

There have been growing numbers of studies of lncRNAs as critical regulators in the initiation and development of PC and biomarkers for PC diagnosis and prognosis. For example, Cheng et al. have reported that depletion of lncRNA SNHG7 restrains PC cell proliferation and metastasis via ID4 by sponging miR-342-3p [19]. Yue et al. have found that lncRNA TUSC7 represses cell proliferation, migration, invasion, EMT, and stemness whereas facilitates apoptosis of PC cells by modulating miR-371a-5p expression [20]. Feng et al. have demonstrated that lncRNA HULC exerts oncogenic roles in PC by downregulating miR-15a and then activating the PI3 K/Akt pathway [21].

In recent years, accumulating evidence has demonstrated that TUG1 is largely overexpressed and serves as an oncogenic gene in many types of cancers, such as colorectal cancer [22], osteosarcoma [23] and prostate cancer [24]. However, in certain cancers, such as non-small cell lung cancer [25] and breast cancer [26], TUG1 has been reported to be expressed at a relatively low level and plays a tumor suppressive role. A study reported by Yin et al. in 2017 shows that compared with other organ tissues, TUG1 is highly expressed in pancreatic tissue, and TUG1 downregulation can affect apoptosis and insulin secretion in pancreatic β cells in vitro and in vivo [27], which may be involved in diabetes pathogenesis. Afterward, Yang and his colleagues have noted the importance of TUG1 in pancreatic ductal adenocarcinoma and pointed out that TUG1 is increased in PC tissues and cells and enhances the viability of PDAC cells and its resistance of gemcitabine [28]. Furthermore, a study reports that TUG1 can function as an oncogene that contributes to tumor progression by competitively sponging miR-382 and thereby regulating EZH2 [29]. Moreover, it is also documented that TUG1 promotes PC cell proliferation and migration via EMT pathway by regulating the TGF- β /Smad signaling pathway [11]. It is demonstrated

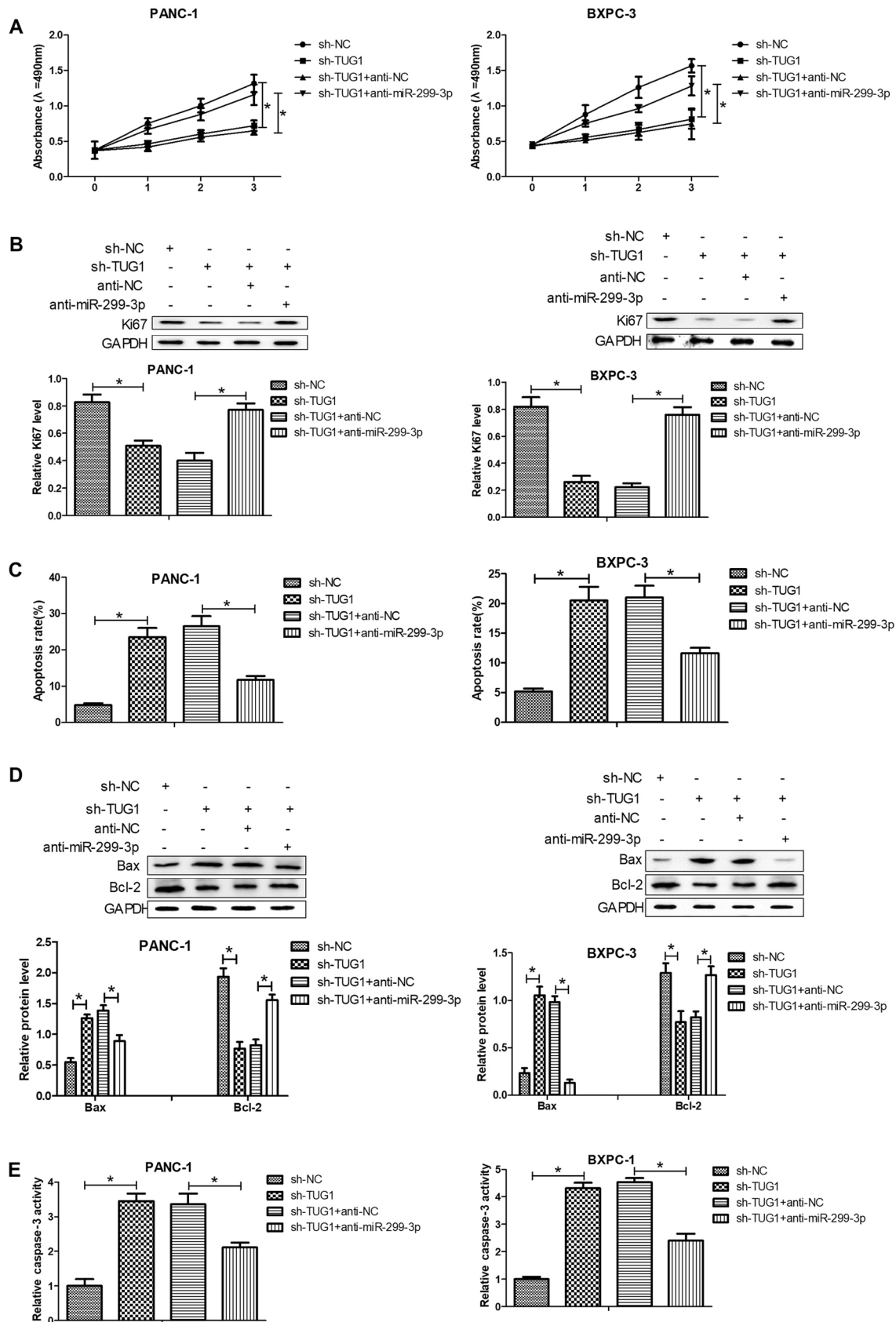


Fig. 3 Effects of TUG1 knockdown or combined with miR-299-3p inhibition on PC cell biological behaviors. **a** Cell proliferation in treated PANC-1 and BXPC-3 cells was evaluated by MTT assay. **b** The protein level of Ki67 in treated PANC-1 and BXPC-3 cells was determined by western blot. **c** Flow cytometry analysis was carried out to detect the apoptotic rate of treated PANC-1 and BXPC-3 cells. **d** The protein level of Bax and Bcl-2 in treated PANC-1 and BXPC-3 cells was examined by western blot. **e** Caspase-3 activity in treated PANC-1 and BXPC-3 cells was measured by caspase-3 activity assay. * $P < 0.05$

that TUG1 silencing leads to a significant suppression of the proliferation and invasion of PC cells in vitro and in vivo by increasing miR-29c expression [10]. All these reported studies show that the abnormal expression of TUG1 functions as a crucial component of complex regulatory network in PC development and progression. In accordance with these previous studies, our study confirmed that TUG1 expression was upregulated in PC tissues, especially in PC patients at an advanced stage 3/4, as well as PC cell lines. And we first found that the increased TUG1 was closely with the advanced stage of PC patients and lymphatic metastasis. These results suggest that TUG1 play crucial roles in PC development. Besides, TUG1 knockdown significantly suppressed cell proliferation, invasion, migration, and EMT, and induced apoptosis in PC cells in vitro, as well as repressed PC tumor growth and EMT in vivo, suggesting the oncogenic role of TUG1 in PC.

To further figure out the molecular mechanism of the oncogenic role of TUG1 in PC, we performed bioinformatics online analysis and luciferase reporter assays. Our results confirmed that TUG1 directly targeted miR-299-3p. Furthermore, our qRT-PCR analyses disclosed that knockdown of TUG1 could be partially inversed by anti-miR-299-3p treatment in PANC-1 and BXPC-3 cells, and knockdown of TUG1 significantly enhanced the expression of miR-299-3p, which manifested for the first time that TUG1 served as a ceRNA for miR-299-3p. As one of the important miRNAs, aberrantly expressed miR-299-3p is reported to participate in the tumorigenesis of several types of cancers. For example, it is discovered that miR-299-3p is underexpressed and functions as a tumor suppressive miRNA in thyroid cancer (TC) [30], hepatocellular carcinoma (HCC) [31], and colon cancer [32]. On the contrary, miR-299-3p is found to be highly expressed in ovarian cancer and miR-299-3p downregulation

inhibits the proliferation by induction of apoptosis, as well as suppresses migration and invasion of ovarian cancer cells [33]. However, the expression and specific function of miR-299-3p in PC cells remains unclear. Herein, we provided the evidence that miR-299-3p was underexpressed in PC tissues, particularly in advanced PC patients, and PC cells. Interestingly, a negative correlation between TUG1 and miR-299-3p expression was observed in PC tissues. Moreover, miR-299-3p inhibition dramatically reversed the effects of TUG1 knockdown on the proliferation, apoptosis, invasion, migration, and EMT in PC cells in vitro and on PC tumor growth and EMT in vivo, suggesting that TUG1 knockdown suppressed PC malignant progression by sponging miR-299-3p.

The Notch1 pathway, an evolutionarily conserved pathway throughout the animal kingdom, is implicated in various physical and pathological biological processes, such as cell differentiation, proliferation, and survival [34]. Activation of the Notch1 pathway has been commonly observed in many human malignancies including PC [35, 36]. The Notch pathway components and its downstream targets such as Survivin and CyclinD1 proteins are upregulated in PC [36]. It is widely believed that abnormal activation of the Notch1 pathway is associated with the carcinogenesis of PC [37, 38]. Moreover, activation of the Notch pathway contributes to the failure of treatment in PC [39]. Accordingly, these results suggest that blockage of the Notch pathway may be a promising treatment method for PC [40]. In our study, we found that TUG1 silencing inhibited the protein levels of Notch1, Survivin, and CyclinD1 in PC cells and PC xenografted tumor tissues, which were abrogated by inhibition of miR-299-3p, suggesting that TUG1 silencing inhibited the activation of Notch1 pathway in PC by upregulating miR-299-3p. Collectively, we concluded that inhibition of TUG1/miR-299-3p axis suppressed PC malignant progression via suppression of the Notch1 pathway.

In summary, our study demonstrated for the first time that TUG1 knockdown inhibited PC malignant progression by sponging miR-299-3p via suppression of Notch1 pathway. Our findings highlighted the TUG1/miR-299-3p/Notch1 signaling in the progression of PC, which may be important for developing novel effective diagnostic and therapeutic strategies for PC.

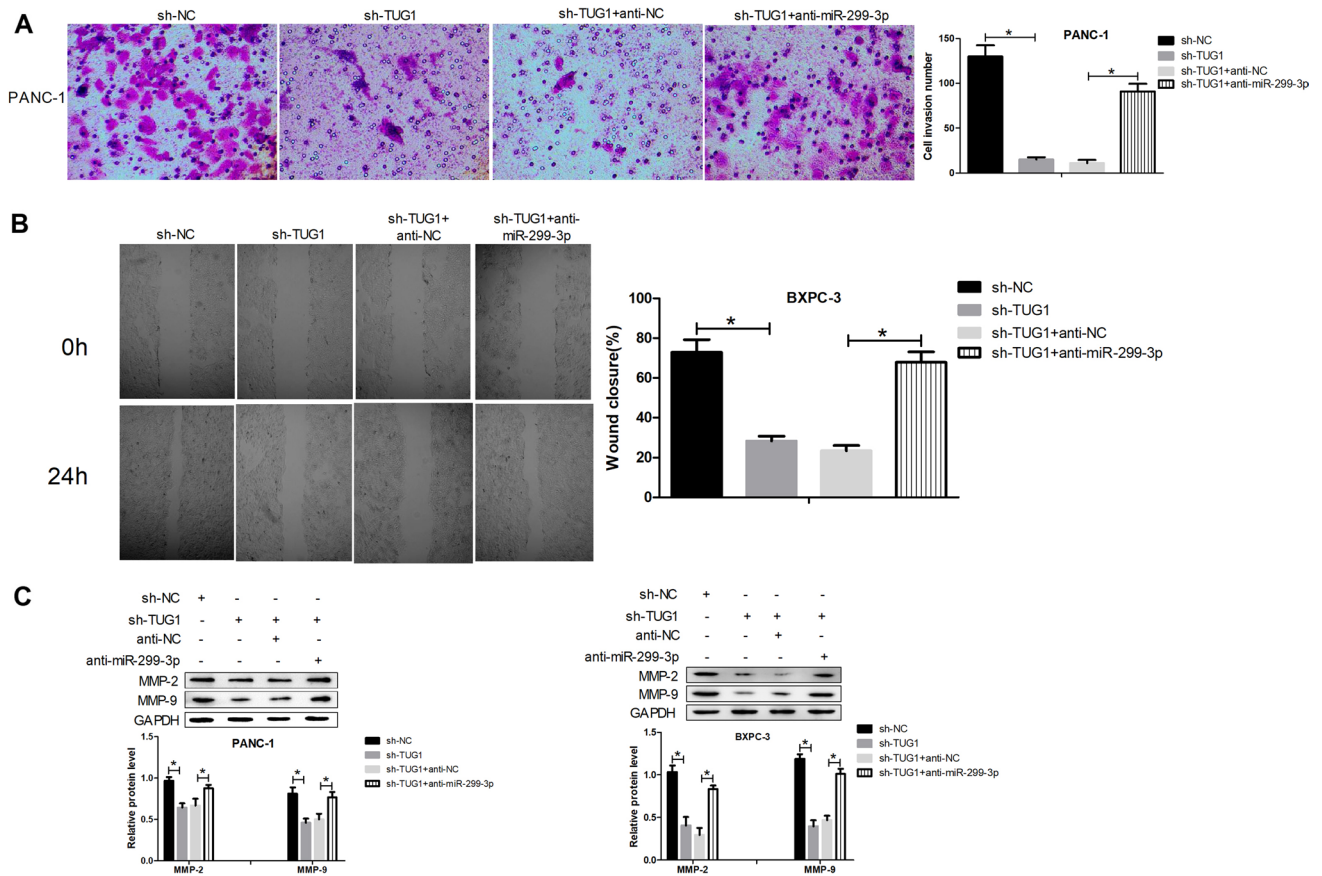


Fig. 4 Effects of TUG1 knockdown or combined with miR-299-3p inhibition on cell invasion and migration of PC cells. **a** Transwell invasion assay was conducted to evaluate cell invasive capacity in treated PANC-1 cells. **b** Wound healing assay was performed to

determine cell migration in treated BXPC-3 cells. **c** Western blot analysis was performed to detect the protein levels of MMP-2 and MMP-9 in treated PANC-1 and BXPC-3 cells. * $P < 0.05$

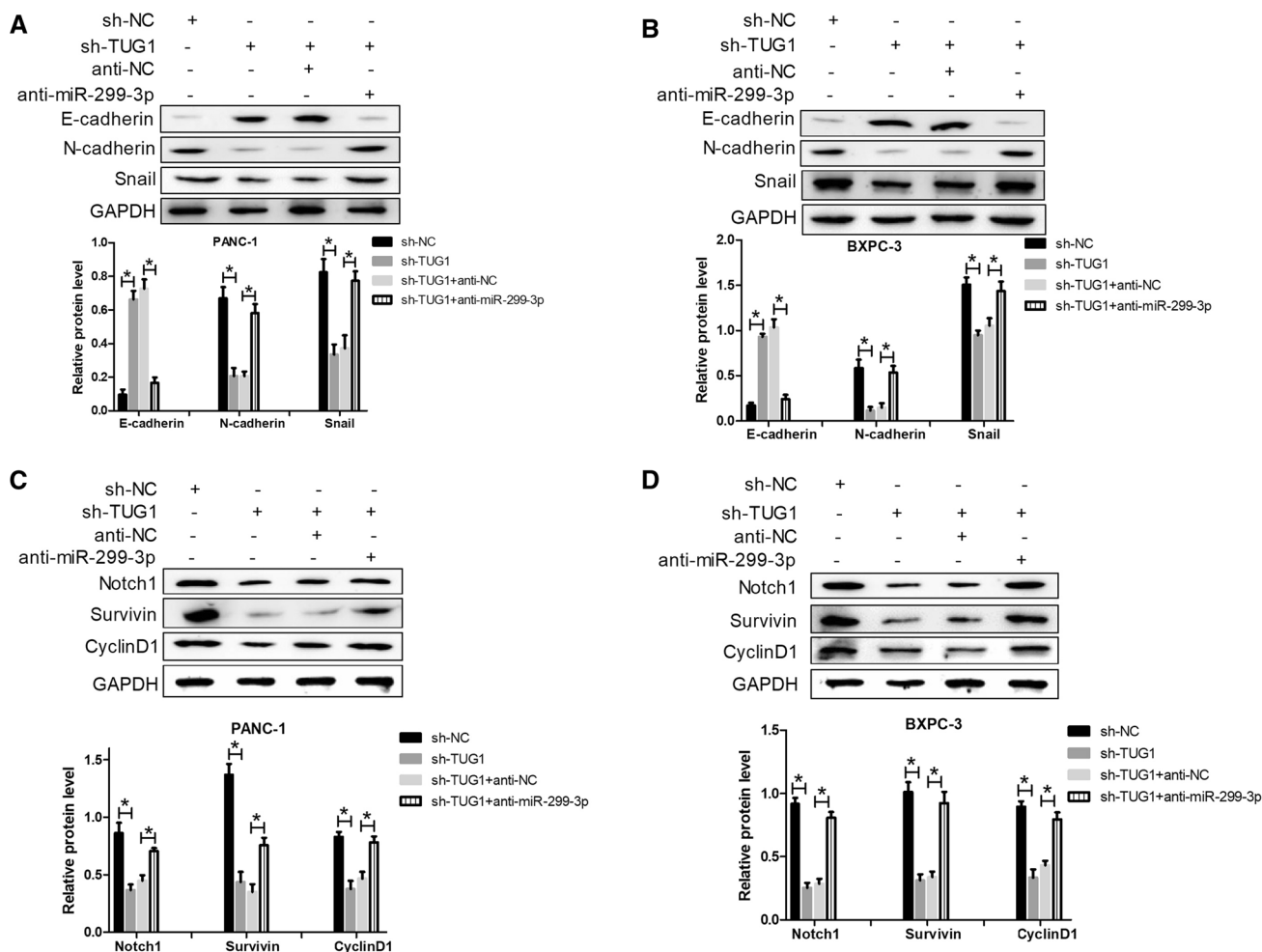


Fig. 5 Effects of TUG1 knockdown or miR-299-3p inhibition on EMT and the Notch1 pathway in PC cells. **a, b** The protein levels of E-cadherin, N-cadherin, and Snail in the treated PANC-1 and

BXPC-3 cells were detected by western blot analysis. **c, d** The protein levels of Notch1, Survivin, and CyclinD1 in the treated PANC-1 and BXPC-3 cells were measured by western blot analysis. **P* < 0.05

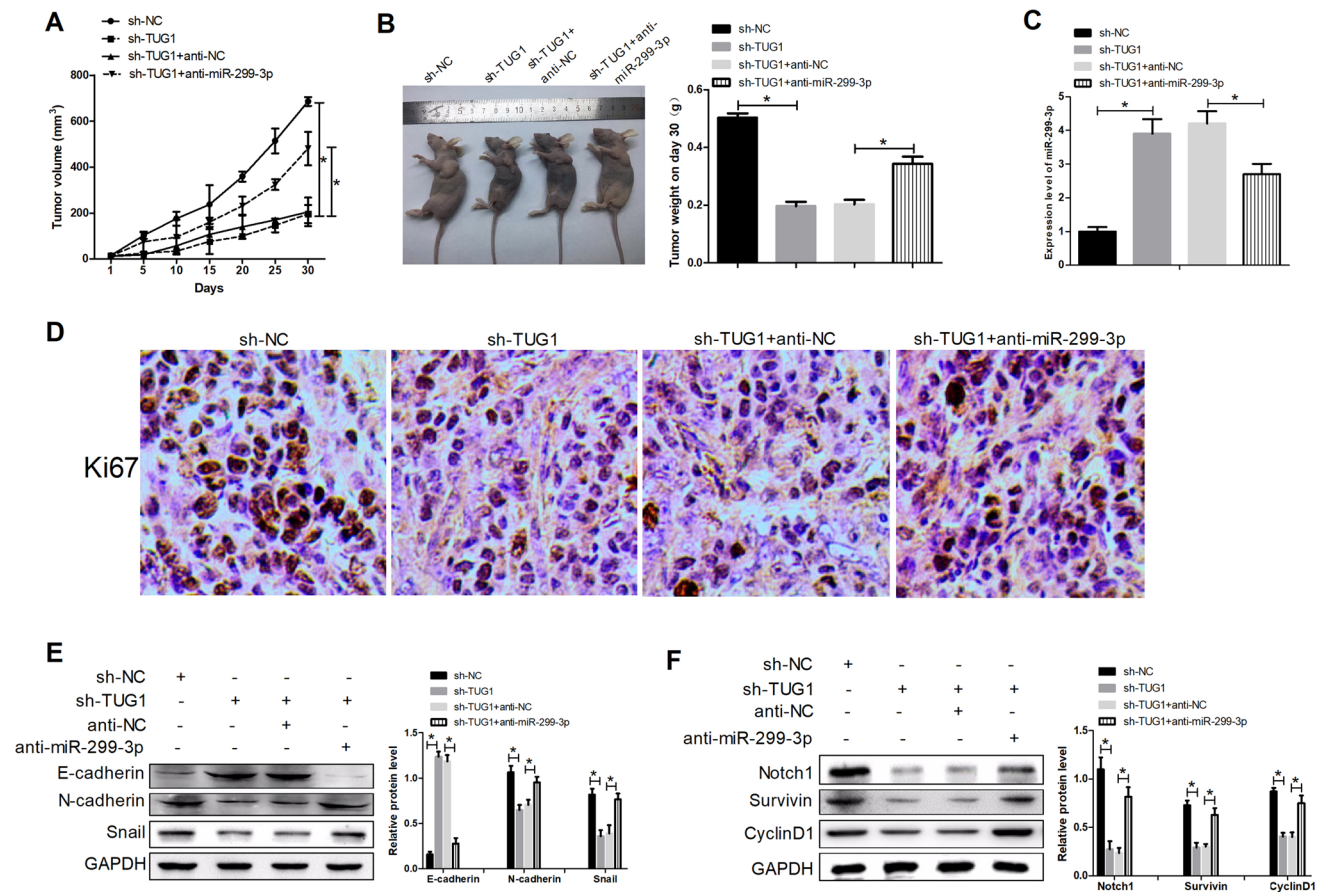


Fig. 6 Effects of TUG1 or combined with miR-299-3p on PC tumor growth, EMT and the Notch1 pathway. PANC-1 cells transfected with sh-TUG1, sh-NC, or combined with anti-miR-299-3p or anti-NC were subcutaneously injected into left subaxillary of the mice to establish the xenograft tumor model. **a** Tumor volume was routinely recorded every 5 days. **b** The tumor-bearing mice were killed at day 30 post-inoculation, and tumors were surgically removed and

weighed. **c** miR-299-3p expression in the excised tumor tissues was detected by qRT-PCR. **d** Ki67 expression in the excised tumor tissues was examined by IHC assay. **e** The expressions of E-cadherin, N-cadherin, and Snail in the excised tumor tissues were detected by western blot analysis. **f** The expressions of Notch1, Survivin, and CyclinD1 in the excised tumor tissues were detected by western blot analysis. * $P < 0.05$

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Compliance with Ethical Standards

Conflict of interest The authors declare that they have no conflict of interest.

References

- Garrido-Laguna I, Hidalgo M. Pancreatic cancer: from state-of-the-art treatments to promising novel therapies. *Nat Rev Clin Oncol*. 2015;12(6):319–334.
- Siegel RL, Miller KD, Jemal A. Cancer statistics. *CA Cancer J Clin*. 2018;68(1):7–30.
- Li D, Xie K, Wolff R, et al. Pancreatic cancer. *Lancet*. 2004;363(9414):1049–1057.

- Schmitt AM, Chang HY. Long noncoding RNAs in cancer pathways. *Cancer Cell*. 2016;29(4):452–463.
- Li J, Meng H, Bai Y, et al. Regulation of lncRNA and its role in cancer metastasis. *Oncol Res*. 2016;23(5):205–217.
- Evans JR, Feng FY, Chinnaiyan AM. The bright side of dark matter: lncRNAs in cancer. *J Clin Invest*. 2016;126(8):2775–2782.
- Khalil AM, Guttman M, Huarte M, et al. Many human large intergenic noncoding RNAs associate with chromatin-modifying complexes and affect gene expression. *Proc Natl Acad Sci USA*. 2009;106(28):11667–11672.
- Li Z, Shen J, Chan MT, et al. TUG1: a pivotal oncogenic long noncoding RNA of human cancers. *Cell Prolif*. 2016;49(4):471–475.
- Hui B, Xu Y, Zhao B, et al. Overexpressed long noncoding RNA TUG1 affects the cell cycle, proliferation, and apoptosis of pancreatic cancer partly through suppressing RND3 and MT2A. *Oncotargets Ther*. 2019;12:1043–1057.
- Lu Y, Tang L, Zhang Z, et al. Long noncoding RNA TUG1/miR-29c axis affects cell proliferation, invasion, and migration in human pancreatic cancer. *Dis Mak*. 2018;2018:6857042.

11. Qin CF, Zhao FL. Long non-coding RNA TUG1 can promote proliferation and migration of pancreatic cancer via EMT pathway. *Eur Rev Med Pharmacol Sci.* 2017;21(10):2377–2384.
12. Krol J, Loedige I, Filipowicz W. The widespread regulation of microRNA biogenesis, function and decay. *Nat Rev Genet.* 2010;11(9):597–610.
13. Iorio MV, Croce CM. MicroRNAs in cancer: small molecules with a huge impact. *J Clin Oncol.* 2009;27(34):5848–5856.
14. Cesana M, Cacchiarelli D, Legnini I, et al. A long noncoding RNA controls muscle differentiation by functioning as a competing endogenous RNA. *Cell.* 2011;147(2):358–369.
15. Liu X, He M, Hou Y, et al. Expression profiles of microRNAs and their target genes in papillary thyroid carcinoma. *Oncol Rep.* 2013;29(4):1415–1420.
16. He H, Wang L, Zhou W, et al. MicroRNA expression profiling in clear cell renal cell carcinoma: identification and functional validation of key miRNAs. *PLoS One.* 2015;10(5):e0125672.
17. Cheng YU, Li H, Li J, et al. O-GlcNAcylation enhances anaplastic thyroid carcinoma malignancy. *Oncol Lett.* 2016;12(1):572–578.
18. Katsushima K, Natsume A, Ohka F, et al. Targeting the Notch-regulated non-coding RNA TUG1 for glioma treatment. *Nat Commun.* 2016;7:13616.
19. Cheng D, Fan J, Ma Y, et al. LncRNA SNHG7 promotes pancreatic cancer proliferation through ID4 by sponging miR-342-3p. *Cell Biosci.* 2019;9:28.
20. Yue L, Guo J. LncRNA TUSC7 suppresses pancreatic carcinoma progression by modulating miR-371a-5p expression. *J Cell Physiol.* 2019;1(2):1–3. <https://doi.org/10.1002/jcp.28248>.
21. Feng H, Wei B, Zhang Y. Long non-coding RNA HULC promotes proliferation, migration and invasion of pancreatic cancer cells by down-regulating microRNA-15a. *Int J Biol Macromol.* 2019;126:891–898.
22. Wang L, Zhao Z, Feng W, et al. Long non-coding RNA TUG1 promotes colorectal cancer metastasis via EMT pathway. *Oncotarget.* 2016;7(32):51713–51719.
23. Zhang Q, Geng PL, Yin P, et al. Down-regulation of long non-coding RNA TUG1 inhibits osteosarcoma cell proliferation and promotes apoptosis. *Asian Pac J Cancer Prev.* 2013;14(4):2311–2315.
24. Yang XL, Wei C, Zhang YB, et al. Long noncoding RNA TUG1 promotes progression via upregulating DGCR1 in prostate cancer. *Eur Rev Med Pharmacol Sci.* 2019;23(6):2391–2398.
25. Zhang EB, Yin DD, Sun M, et al. P53-regulated long non-coding RNA TUG1 affects cell proliferation in human non-small cell lung cancer, partly through epigenetically regulating HOXB7 expression. *Cell Death Dis.* 2014;5:e1243.
26. Fan S, Yang Z, Ke Z, et al. Downregulation of the long non-coding RNA TUG1 is associated with cell proliferation, migration, and invasion in breast cancer. *Biomed Pharmacother.* 2017;95:1636–1643.
27. Yin DD, Zhang EB, You LH, et al. Downregulation of lncRNA TUG1 affects apoptosis and insulin secretion in mouse pancreatic beta cells. *Cell Physiol Biochem.* 2015;35(5):1892–1904.
28. Yang F, Li X, Zhang L, et al. LncRNA TUG1 promoted viability and associated with gemcitabine resistant in pancreatic ductal adenocarcinoma. *J Pharmacol Sci.* 2018;137(2):116–121.
29. Zhao L, Sun H, Kong H, et al. The Lncrna-TUG1/EZH2 axis promotes pancreatic cancer cell proliferation, migration and EMT phenotype formation through sponging Mir-382. *Cell Physiol Biochem.* 2017;42(6):2145–2158.
30. Chen X, Qi M, Yang Q, et al. MiR-299-3p functions as a tumor suppressor in thyroid cancer by regulating SHOC2. *Eur Rev Med Pharmacol Sci.* 2019;23(1):232–240.
31. Dang S, Zhou J, Wang Z, et al. MiR-299-3p functions as a tumor suppressor via targeting Sirtuin 5 in hepatocellular carcinoma. *Biomed Pharmacother.* 2018;106:966–975.
32. Wang JY, Jiang JB, Li Y, et al. MicroRNA-299-3p suppresses proliferation and invasion by targeting VEGFA in human colon carcinoma. *Biomed Pharmacother.* 2017;93:1047–1054.
33. Zhao R, Liu Q, Lou C. MicroRNA-299-3p regulates proliferation, migration and invasion of human ovarian cancer cells by modulating the expression of OCT4. *Arch Biochem Biophys.* 2018;651:21–27.
34. Gordon WR, Arnett KL, Blacklow SC. The molecular logic of Notch signaling—a structural and biochemical perspective. *J Cell Sci.* 2008;121(Pt 19):3109–3119.
35. Takebe N, Harris PJ, Warren RQ, et al. Targeting cancer stem cells by inhibiting Wnt, Notch, and Hedgehog pathways. *Nat Rev Clin Oncol.* 2011;8(2):97–106.
36. Miyamoto Y, Maitra A, Ghosh B, et al. Notch mediates TGF alpha-induced changes in epithelial differentiation during pancreatic tumorigenesis. *Cancer Cell.* 2003;3(6):565–576.
37. Hu H, Zhou L, Awadallah A, et al. Significance of Notch1-signaling pathway in human pancreatic development and carcinogenesis. *Appl Immunohistochem Mol Morphol.* 2013;21(3):242–247.
38. Gao J, Long B, Wang Z. Role of Notch signaling pathway in pancreatic cancer. *Am J Cancer Res.* 2017;7(2):173–186.
39. Lee JY, Song SY, Park JY. Notch pathway activation is associated with pancreatic cancer treatment failure. *Pancreatol.* 2014;14(1):48–53.
40. Mysliwiec P, Boucher MJ. Targeting Notch signaling in pancreatic cancer patients—rationale for new therapy. *Adv Med Sci.* 2009;54(2):136–142.

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