Original Paper

The Lncrna-TUG1/EZH2 Axis Promotes Pancreatic Cancer Cell Proliferation, Migration and EMT Phenotype Formation Through Sponging Mir-382

Liang Zhao^a Hongwei Sun^b Hongru Kong^b Zongjing Chen^b Bicheng Chen^a Mengtao Zhou^b

^aKey Laboratory of Surgery, The First Affiliated Hospital, Wenzhou Medical University, 2 FuXue Lane, Wenzhou, Zhejiang, ^bDepartment of Surgery, The First Affiliated Hospital, Wenzhou Medical University, 2 FuXue Lane, Wenzhou, Zhejiang, China

Key Words

Lncrna-TUG1 • MiR-382 • EZH2 • Pancreatic cancer • Proliferation • Migration • EMT

Abstract

Background/Aims: Pancreatic carcinoma (PC) is the one of the most common and malignant cancers worldwide. LncRNA taurine upregulated gene 1 (TUG1) was initially identified as a transcript upregulated by taurine, and the abnormal expression of TUG1 has been reported in many cancers. However, the biological role and molecular mechanism of TUG1 in PC still needs further investigation. **Methods:** Quantitative real-time PCR (qRT-PCR) was performed to measure the expression of TUG1 in PC cell lines and tissues. MTT and colony formation assays were used to measure the effect of TUG1 on cell proliferation. A wound healing assay, transwell assay and western blot assay were employed to determine the effect of TUG1 on cell migration and the epithelial mesenchymal transition (EMT) phenotype. RNA-binding protein immunoprecipitation (RIP) and a biotin-avidin pulldown system were performed to confirm the interaction between miR-328 and TUG1. A gene expression array analysis using clinical samples and RT-qPCR suggested that enhancer of zeste homolog 2 (EZH2) was a target of miR-382 in PC. **Results:** In this study, we reported that TUG1 was overexpressed in PC tissues and cell lines, and high expression of TUG1 predicted poor prognosis. Further experiments revealed that overexpressed TUG1 promoted cell proliferation, migration and contributed to EMT formation, whereas silenced TUG1 led to opposing results. Additionally, luciferase reporter assays, an RIP assay and an RNA-pulldown assay demonstrated that TUG1 could competitively sponge miR-382 and thereby regulate EZH2. **Conclusion:** Collectively, these findings revealed that TUG1 functions as an oncogenic lncRNA that promotes tumor progression, at least partially, by functioning as an endogenous 'sponge' and competing for miR-382 binding to the miRNA target EZH2.

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Mengtao Zhou

Department of Surgery, The First Affiliated Hospital, Wenzhou Medical University,
2 FuXue Lane, Wenzhou, Zhejiang (China)
E-Mail zmt0417@hotmail.com

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Introduction

Pancreatic cancer (PC) is one of the deadliest human malignancies and the fourth-leading cause of cancer-related deaths [1, 2]. Due to a lack of efficient methods for early diagnosis, patients are always diagnosed at an advanced stage. Currently, surgical resection is an important treatment for PC; however, only 10-20% of patients are diagnosed at a resectable stage and the 5-year survival of post-operation for PC is still unsatisfactory at only 20% [3]. Thus, chemotherapy becomes one of the indispensable means of clinical treatment for PC. Early metastasis is one of the characteristics of pancreatic cancer, which always results in a poor curative effect or chemotherapy failure. Epithelial-to-mesenchymal (EMT), a process of losing epithelial phenotypes and gaining mesenchymal phenotypes, contributes to the early dissemination of primary tumor cells and metastatic spread [4-6]. The aim of our present study is to explore the underlying EMT formation mechanism of PC and potential therapeutic value.

Long non-coding RNAs (lncRNAs), defined as non-coding RNA more than 200 nt with limited or no protein coding ability, are gaining prominence. The function of lncRNAs has been characterized in many cancers [7]. lncRNAs exert their function mainly at three levels: transcription, post-transcription and epigenetic modification. Taurine-upregulated gene 1 (TUG1), initially identified as a transcript upregulated in response to taurine treatment of developing mouse retinal cells, is aberrantly expressed in many human cancers and involved in carcinogenesis and progression [8-11].

In our present study, we explored the role of TUG1 in the regulation of proliferation, migration and EMT formation of PC. Our findings show that silencing TUG1 impairs migration and epithelial mesenchymal transition ability. Moreover, for the first time, we identify an important role for TUG1 in pancreatic cancer cells. Furthermore, we demonstrate that TUG1 could competitively bind miR-382 and then regulate the expression of EZH2, thereby affecting the progression of PC.

Materials and Methods

Patients

Pancreatic samples were obtained from the Department of Surgery, The First Affiliated Hospital, Wenzhou Medical University. None of the patients received chemotherapy or radiotherapy before the surgery. All samples were frozen in liquid nitrogen immediately after resection and stored at -80 °C until use. This study was approved by the institutional review boards at all of the hospitals involved in the study, and written informed consent was obtained from all patients.

Cell lines

Human PDAC cell lines PANC-1, AsPC-1 and HEK 293T cells were obtained from ATCC (the American Type Culture Collection, Manassas, VA, USA). PATU 8988, BxPC-3, SW 1990 cells and normal pancreatic cells HPDE6-C7 were purchased from the Institute of Biochemistry and Cell Biology of the Chinese Academy of Sciences (Shanghai, China). All cell lines were cultured at 37 °C in a 5% CO₂ humidified atmosphere in DMEM or RPMI 1640 (Gibco, Carlsbad, USA) with 10% fetal bovine serum (FBS, HyClone).

Cell cytoplasm/nucleus fraction isolation

NE-PER Nuclear and Cytoplasmic Extraction Reagents (Thermo Scientific, Waltham, MA, USA) were employed to prepare cytoplasmic and nuclear extracts from PDAC cells. RNA extracted from each of the fractions was subjected to RT-qPCR analysis to demonstrate the levels of nuclear control transcript (MALAT1), cytoplasmic control transcript (GAPDH), lncRNA TUG1, and miR-382.

Quantitative real time PCR Analyses

Total RNA was extracted from the tissues or cultured cells using TRIzol reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. Reverse transcription was performed with the

PrimeScript RT reagent Kit (Takara, Dalian, Liaoning, China). qRT-PCR was performed with SYBR Prime Script RT-PCR Kits (Takara, Japan) based on the manufacturer's instructions. The target gene expression level was calculated using the $2^{-\Delta\Delta Ct}$ method, which was normalized to GAPDH mRNA. All assays were performed in triplicate. The expression levels were relative to the fold change of the corresponding controls, which were defined as 1.0.

Cell viability

Cell viability was assessed via 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyl-tetrazolium bromide (MTT) assay; 5×10^3 cells/well were seeded in a 96-well flat-bottomed plate for 24 h, then transfected with corresponding sh-RNA or pc-DNA3.1 and cultured in normal medium. At 0, 24, 48, 72 and 96 h after transfection, the MTT solution (5 mg/ml, 20 μ l) was added to each well. After incubation for 4 h, the media was removed and 100 μ l DMSO was added to each well. The relative number of surviving cells was assessed by measuring the optical density (OD) of cell lysates at 560 nm. All assays were performed in triplicate.

Colony formation assay

The indicated cells were plated into 6-well plates (1×10^3 cells/well) and incubated in DMEM or RPMI 1640 with 10% FBS at 37 °C. Two weeks later, the cells were washed with PBS, fixed in methanol for 30 min and stained with 1% crystal violet dye, and the number of colonies was counted. All assays were performed in triplicate.

Cell migration and invasion assays

Cell migration was measured by transwell chamber (8 μ m pore size, Corning). Forty-eight hours after transfection, cells in serum-free media were placed into the upper chamber. Media containing 10% FBS was added into the lower chamber. Following 48 h incubation, cells remaining in the upper membrane were wiped off while cells that migrated were fixed in methanol, stained with 0.1% crystal violet and counted under a microscope. Three independent experiments were performed.

Wound healing assays

Cell migration capacity was calculated by wound healing assay; 2×10^5 cells with or without transfection were plated into 12-well plates and incubated in DMEM or RPMI 1640 with 10% FBS at 37°C. After reaching 100% confluence, the cells were wounded by scraping with a 200 μ l tip, followed by 3 washes in serum-free medium and incubation in regular medium. Wounds were observed at 0 and 48 h. The cell migration distance was calculated by subtracting the wound width at each time point from the wound width at the 0 h time point. Three independent assays were performed.

Western blots

Cells were lysed in RIPA lysis buffer (P0013, Beyotime), and nuclear proteins were extracted using lysis buffer (P0028, Beyotime). All of the procedures followed the manufacturer's protocol. Subsequently, the cell lysates were boiled in 5X SDS-PAGE loading buffer for 10 min and then resolved by 8% SDS-PAGE and transferred to a nitrocellulose membrane. The following antibodies were used in this study: E-cadherin, N-cadherin, vimentin, EZH2 and β -catenin were purchased from Cell Signaling Technology. GAPDH (Proteintech) was used as the internal reference. Bound antibodies were visualized with an ECL kit (P0018, Beyotime).

Dual luciferase reporter assay

Cells (293 T) were seeded at 3×10^4 cells/well in 24-well plates. The next day, the cells were co-transfected with pmirGLO-TUG1-WT or -MUT reporter plasmids and a miR-382 mimic. Cells were also co-transfected with pmirGLO-EZH2-WT or -MUT reporter plasmids and miR-382 mimic. Twenty-four hours after transfection, the relative luciferase activity was measured using the Dual-Luciferase Reporter Assay System (Promega, Madison, WI, USA) and normalized against Renilla luciferase activity.

RNA-binding protein immunoprecipitation

RIP experiments were performed using the Magna RIP RNA-binding protein immunoprecipitation kit (Millipore, Billerica, MA, USA) and the Ago2 antibody (Abcam, Cambridge, MA, USA) following the manufacturer's protocol. Co-precipitated RNAs were subjected to RT-qPCR analysis.

RNA-Pulldown assay

PANC-1 cells were transfected with biotinylated miRNA and collected 48 h after transfection. The cell lysates were incubated with M-280 streptavidin magnetic beads (Invitrogen, San Diego, CA, USA). The bound RNAs were purified using TRIzol reagent (Invitrogen) for further RT-qPCR analysis.

Statistical analyses

Statistical analyses were performed using SPSS 17.0 software (SPSS, Chicago, IL, USA). Differences between two groups were assessed using Student's t-test (two-tailed). Correlations between TUG1 and miR-382 or EZH2 were analyzed using Spearman rank correlation. Each experiment was performed at least three times. The results of the experiments are displayed as the mean $\pm$ SD. A P-value <0.05 indicated statistical significance.

Results

TUG1 is upregulated in PC and correlated with clinical outcome of pancreatic cancer patients

To study the lncRNA mechanism involved in regulating the progression of PC, we previously conducted a lncRNA expression array analysis using clinical samples (Fig. 1A). Among those lncRNAs, lncRNA-PVT1 and lncRNA-TUG1 were highly expressed in pancreatic

Fig. 1. TUG1 is upregulated in PC and correlated with clinical outcome of pancreatic cancer patients. (A) lncRNA expression array analysis of clinical samples. (B) TUG1 expression in paired samples of PC and adjacent normal tissues. (C) The overall survival in 34 pancreatic cancer (PC) patients was represented by Kaplan-Meier curves. (D) Relative levels of TUG1 in normal HPDE6-C7 cells and five types of PC cells. The p-value represents the comparison between groups (* $p<0.05$, ** $p<0.01$).

tissues. The biology function of PVT1 has been discussed in our previous study. In the present study, we focus on the function of TUG1. To further validate the expression level of TUG1, we evaluated the TUG1 expression level in 34 paired PC and adjacent pancreatic tissue samples using reverse transcription and quantitative real-time PCR (RT-qPCR). The results showed that TUG1 levels were significantly higher in tumor tissues (Fig. 1B). Additionally, correlation between lnc-TUG1 expression and clinical features (Table 1) revealed that a high level of TUG1 was correlated with large tumor size ($p=0.000$), poor tumor differentiation ($p=0.000$), TNM stage ($p=0.001$), vascular infiltration ($p=0.000$), and distant metastasis ($p=0.000$) and negatively correlated with overall survival (OS) of patients with pancreatic cancer (Fig. 1C), which indicated that upregulated TUG1 might contribute to the development of pancreatic cancer. Then, we examined the expression level of TUG1 in PC cell

Table 1. Correlation between lnc-TUG1 Expression and Clinical Features. (n=34). Low/high by the sample mean. Pearson χ^2 test. $P<0.05$ was considered statistically significant

Variable	Lnc-TUG1 Expression low	Lnc-TUG1 Expression high	P-value
Age			
<60	12	12	0.715
≥60	4	6	
Gender			
Male	13	11	0.270
Female	3	7	
Tumor Size			
<2	14	3	0.000
≥2	2	15	
Histological Grade			
High/Moderate	13	1	0.000
Low	3	17	
TNM Stage			
I-II	11	2	0.001
III-IV	5	16	
Lymphatic invasion			
positive	13	13	0.693
negative	5	5	
Vascular infiltration			
positive	4	17	0.000
negative	12	1	
Distant metastasis			
positive	3	17	0.000
negative	13	1	

lines (PATU 8988, BxPC-3, PANC-1, SW 1990, AsPC-1) and HPDE6-C7 cells, an immortalized, normal human pancreatic epithelial cell line, using RT-qPCR. Compared with HPDE6-C7 cells, PC cells exhibited significantly higher levels of TUG1 expression but not PATU 8988 cells (Fig. 1D). Collectively, these results suggest that TUG1 is upregulated in PC.

TUG1 promotes PC cell proliferation, migration and EMT formation

To evaluate the role of TUG1 in regulating the proliferation, migration and EMT formation, we transfected PATU 8988 and PANC-1 cell lines with a TUG1 expression vector and shRNA/TUG1, respectively (Fig. 2A). First, we performed MTT and colony formation assays to monitor the effect of TUG1 on cell proliferation. We observed a significant increase in proliferation of cells transfected with the TUG1 expression vector compared with the negative control in PATU 8988 cells (Fig. 2B and 2C, left). Furthermore, knockdown expression of TUG1 in PANC-1 cells resulted in a significant increase in cell proliferation (Fig. 2B and 2C, right). Then, we test the effect of TUG1 on cell migration capacity. A wound healing assay showed that increased expression of TUG1 drastically increased the cell migration capacity while TUG1 knockdown significantly decreased the cell migration capacity (Fig. 2D-E). These data provide evidence of the migration-promoting role of TUG1 in vitro. Additionally, we examined the influence of TUG1 on the formation of EMT. The results from western blotting showed that overexpression of TUG1 contributed to the formation of EMT, and vice versa in cells with TUG1 knocked down (Fig. 2F-G). Taken together, these results suggest that TUG1 has important roles in PC cell proliferation, migration and EMT formation. However, the detailed mechanism by which TUG1 functions warrants further investigation.

MiR-382 is regulated by TUG1

In addition to characterizing the function of TUG1 on PC cells, we also sought to explore in further detail the mechanism by which TUG1 exerts its functions. Accumulating evidence identified that many lncRNAs have been reported to function as competing endogenous (ce)

Fig. 2. TUG1 promotes PC cell proliferation, migration and EMT formation. (A) The overexpression and knock-down efficiency of TUG1 expression vector and sh-TUG1 was obtained after transfection 48 h. (B) MTT, (C) colony formation, (D) wound healing assay, (E) transwell assay, and (F) western blot assay were performed in PATU 8988 cells transfected with pcDNA3.1/TUG1or in PANC-1 cells with sh-TUG1. All data were represented as the mean $\pm$ S.D. from three independent experiments, * P <0.05, ** P <0.01.

RNAs by serving as sponges that bind and sequester miRNAs [12-15]. To examine whether TUG1 exerts its function in PC cells through functioning as a ceRNA, we first measured the percentage of TUG1 in the cytoplasmic and nuclear fractions of PANC-1 cells. As shown in Fig. 3A, RT-qPCR results of nuclear and cytoplasmic fractions of PANC-1 cells present that TUG1 was located in the cytoplasm, providing a prerequisite for function as a ceRNA. Then, using starBase software (<http://starbase.sysu.edu.cn/mirLncRNA.php>), we measured the miRNAs predicted to be bound to TUG1 in response to knockdown of TUG1. We transfected PANC-1 cell with shTUG1, and we found that downregulated TUG1 had the most significant effect on miR-382 expression (Table 2). Next, RT-qPCR of nuclear and cytoplasmic fractions of PATU 8988 and PANC-1 cells validated that miR-382 was also located in the cytoplasm, indicating a potential reciprocal interaction between TUG1 and miR-382 (Fig. 3B). Thus, miR-382 was pursued for studies in detail. RT-qPCR showed that, in PC tissues, miR-382 was downregulated in the tumor specimens, which was inversely correlated with TUG1 (Fig. 3C). Compared with HPDE6-C7 cells, PC cell lines displayed lower expression of miR-382, except for in PATU 8988 cells (Fig. 3D). To further evaluate the potential regulating relationship between TUG1 and miR-382, PATU 8988 and PANC-1 cells were transfected with a TUG1 expression vector and shRNA/TUG1 to increase or decrease TUG1 expression, respectively. Overexpression of TUG1 resulted in a significant downregulation of miR-382 while knockdown of TUG1 significantly increased the level of miR-382 (Fig. 3E). These results suggest that miR-382 was negatively regulated by TUG1. To further explore whether TUG1-mediated miR-382 regulation through functioning as a ceRNA, we performed a dual luciferase reporter assay, RNA-binding protein immunoprecipitation (RIP) and applied a biotin-avidin pulldown system. We first sub-cloned full-length of TUG1 into the pmirGLO dual luciferase reporter vector and performed

Fig. 3. MiR-382 is regulated by TUG1. (A) RT-qPCR detection of the percentage of TUG1, miR-382. (B) GAPDH and U6 in the cytoplasmic and nuclear fractions of PANC-1 cells. GAPDH and U6 serve as a cytoplasmic and nuclear localization marker, respectively. (C) miR-382 expression in paired samples of PC and adjacent normal tissues. (D) Relative levels of miR-382 in normal HPDE6-C7 cells and five types of PC cells. (E) The expression of miR-382 in response to the level of TUG1. (F) Luciferase assays of the indicated cells transfected with pmirGLO-TUG1-WT or pmirGLO-TUG1-MUT reporter and NC or miR-382 mimic. (G) Amount of TUG1 bound to Ago2 or IgG measured by RT-qPCR after RIP; IgG was used as a negative control and SNRNP70 was used as a positive control. (H) PANC-1 cells were transfected with biotinylated WT miR-382 (miR-382-Bio) or biotinylated mutant miR-382 (miR-382-MutBio) or biotinylated NC (NC-Bio). Forty-eight hours after transfection, cells were collected for a biotin-based pulldown assay. TUG1 expression levels were analyzed by RT-qPCR. All data were represented as the mean $\pm$ S.D. from three independent experiments, * $P < 0.05$, ** $P < 0.01$.

luciferase assays in PANC-1 cells (Fig. 3F). As shown in Fig. 3F, co-transfection of PANC-1 cells with pmirGLO-TUG1-WT vector and miR-382 mimic significantly reduced luciferase reporter activity with respect to the negative control while cells co-transfected with pmirGLO-TUG1-MUT vector and negative control showed a higher level of luciferase activity compared to the group of co-transfected with WT vector and negative control. The results from RIP showed that Ago2 protein was efficiently immunoprecipitated from cell extracts by Ago2

antibody (Fig. 3G), which revealed that while TUG1 was detected in Ago2 immunoprecipitates from the control group, its levels were drastically reduced in Ago2 complexes purified from cells treated with miR-382 inhibitor, indicating that TUG1 is likely in the miR-382 RISC complex. Further, a biotin-avidin pulldown system was applied to assay whether miR-382 could pull down TUG1. As shown in Fig. 3H, TUG1 was pulled down by miR-382, but when the binding site of TUG1 for miR-382 was mutated, TUG1 was not pulled down, indicating that TUG1 regulated miR-382 in a sequence-specific manner. Together, our findings revealed that TUG1 exerts inhibitory effects on miR-382 expression through functioning as a ceRNA and therefore directly sponging miR-382.

MiR-382 reversed the function of TUG1 in PC cells

We transfected PATU 8988 with a TUG1 expression vector and miR-382 mimic to study the effects of miR-382 on cell proliferation, migration and EMT mediated by TUG1. MTT and colony formation assays revealed that TUG1 promoted and miR-382 suppressed cell proliferation while co-transfection of the miR-382 mimic and TUG1 expression vector showed that TUG1-promoted cell proliferation was inhibited by miR-382 (Fig. 4A-B). A wound healing assay and transwell assay revealed that TUG1 promoted and miR-382 suppressed cell migration while co-transfection of the miR-382 mimic and TUG1 expression vector showed that TUG1-promoted cell migration was inhibited by miR-382 (Fig. 4C-D). Moreover, the results from a western blot demonstrated that TUG1 promoted and miR-382 suppressed PC cell EMT phenotype formation while co-transfection of miR-382 mimic and TUG1 expression vector showed that miR-382 reversed the EMT promoted by TUG1 to MET (Fig. 4E). These observations suggest that the effects of TUG1 overexpression on the promotion of PC cell migration and EMT formation could be reversed by miR-382 mimics.

EZH2, a target of miR-382, is positively regulated by TUG1

Previously, we conducted a gene expression array analysis using clinical samples, resulting in the identification of several genes (Fig. 5A) that are involved in PC progression and metastasis. To screen the appropriate target of miR-382, we first measured the identified genes expression in the cells treated with miR-382 mimics. As shown in Fig. 5B, miR-382 showed a significant effect on the level of EZH2. RT-qPCR showed that, in PC tissues, EZH2 was upregulated in the tumor specimens, which was positively correlated with TUG1 (Fig. 5C). Additionally, bioinformatics analysis revealed that in the 3'UTR region of EZH2 contains a binding site for miR-382 (Fig. 5D left). Moreover, the results from a dual luciferase reporter

Table 2. Initial profiling of miRNAs

	miRNAs (prediction of binding)	Fold-change (comparison of siRNA knockdown)
1	hsa-miR-186-5p	0.6694
2	hsa-miR-197-3p	1.0253
3	hsa-miR-9-5p	0.9612
4	hsa-miR-29c-3p	0.6923
5	hsa-miR-29b-3p	0.8016
6	hsa-miR-194-5p	0.9112
7	hsa-miR-196a-5p	0.9663
8	hsa-miR-370-3p	1.2256
9	hsa-miR-299-3p	0.4368
10	hsa-miR-376a-3p	0.7695
11	hsa-miR-376b-3p	0.2298
12	hsa-miR-300	0.7695
13	hsa-miR-381-3p	0.4676
14	hsa-miR-382	8.1046
15	hsa-miR-410-3p	1.3925
16	hsa-miR-365a-3p	1.1035
17	hsa-miR-138-5p	0.7346
18	hsa-miR-328-3p	0.5693
19	hsa-miR-132-3p	1.2154
20	hsa-miR-212-3p	1.5487
21	hsa-miR-144-3p	2.1154
22	hsa-miR-142-3p	0.9675
23	hsa-miR-21-5p	1.5486
24	hsa-miR-125a-3p	2.5589
25	hsa-miR-216b-5p	1.9936
26	hsa-miR-4782-3p	1.2364
27	hsa-miR-153-3p	2.4484
28	hsa-miR-425-5p	0.6689
29	hsa-miR-145	1.2148
30	hsa-miR-219a-5p	0.7695
31	hsa-miR-196b-5p	1.2448
32	hsa-miR-590-5p	0.6645
33	hsa-miR-335-5p	2.4452
34	hsa-miR-29a-3p	0.9663
35	hsa-miR-31-5p	1.2443
36	hsa-miR-221-3p	2.4485
37	hsa-miR-222-3p	0.8516
38	hsa-miR-421	0.7046
39	hsa-miR-542-3p	1.9632
40	hsa-miR-505-3p	2.4513

Fig. 4. MiR-382 reversed the function of TUG1 in PC cells. (A) Cell MTT assay, (B) colony formation assay, (C) wound healing assay, (D) transwell assay (E) and western blot were performed in PATU 8988 cells transfected with miR-NC+NC, miR-NC+TUG1, NC+miR-382 and miR-382+TUG1. All data were represented as the mean $\pm$ S.D. from three independent experiments, * P <0.05, ** P <0.01.

assay showed that co-transfection of PANC-1 cells with the pmirGLO-EZH2-WT vector and miR-382 mimic significantly reduced luciferase reporter activity. In contrast, cells co-transfected with the pmirGLO-EZH2-MUT vector and negative control showed a higher level of luciferase activity (Fig. 5D right). Furthermore, PATU 8988 and PANC-1 cells were transfected with miR-382 mimics and miR-382 inhibitor to decrease or increase miR-382 expression, respectively. Overexpression of miR-382 led to a significant downregulation of EZH2 while knockdown of miR-382 significantly increased the level of EZH2 both in mRNA and protein levels (Fig. 5E). Additionally, the results from RT-qPCR and western blot revealed that overexpression of TUG1 increased the level of EZH2 while downregulation of TUG1 led to reduction of EZH2 both in miRNA and protein levels (Fig. 5F). Collectively, these findings suggest that EZH2 is a target of miR-382 and is positively regulated by TUG1.

Knockdown of EZH2 abolished the function of TUG1

We transfected PATU898 with a TUG1 expression vector and sh-EZH2 to study the effects of sh-EZH2 on cell migration and EMT mediated by TUG1. MTT and colony formation assays revealed that TUG1 promoted and sh-EZH2 suppressed cell proliferation while co-

Fig. 5. EZH2, a target of miR-382, is positively regulated by TUG1. (A) Gene expression array analysis of clinical samples. (B) MiR-382 showed significant effect on the level of EZH2. (C) EZH2 expression in paired samples of PC and adjacent normal tissues. (D) Luciferase assays of the indicated cells transfected with pmirGLO-EZH2-WT or pmirGLO-EZH2-MUT reporter and NC or miR-382 mimic. (E) The expression level of EZH2 both in mRNA and protein level in response to the level of TUG1 and miR-382 (F) was measured. All data were represented as the mean $\pm$ S.D. from three independent experiments, * P <0.05, ** P <0.01.

transfection of sh-EZH2 and TUG1 expression vector showed that TUG1 promoted cell proliferation inhibited by sh-EZH2 (Fig. 6A-B). A wound healing assay and transwell assay revealed that TUG1 promoted and sh-EZH2 suppressed cell migration while co-transfection of sh-EZH2 and TUG1 expression vector showed that TUG1 promoted cell migration inhibited by sh-EZH2 (Fig. 6C-D). Moreover, the results from western blots demonstrated that TUG1 promoted and sh-EZH2 suppressed PC cell EMT phenotype formation while co-transfection of sh-EZH2 and the TUG1 expression vector showed that sh-EZH2 reversed the EMT promoted by TUG1 to MET (Fig. 6E). These findings suggest that decreased expression of EZH2 inhibited PC cell migration and reversed EMT formation.

The expression level of TUG1 was negatively correlated with miR-382 and positively correlated with EZH2 in PC tissues

Finally, the relationship of the three molecules was analyzed. As shown in Fig. 7A, miR-382 levels and TUG1 levels were negatively correlated (2-tailed Spearman's correlation, $r = -0.421$, $p < 0.01$). Additionally, the level of EZH2 showed a positive correlation with TUG1 levels (2-tailed Spearman's correlation, $r = 0.697$, $p < 0.01$; Fig. 7B). Collectively, these data indicated that there is a regulatory signaling pathway in which TUG1 regulates EZH2 by competitively sponging miR-382, inducing increased migration capacity and enhanced EMT phenotype formation of PC cells.

Fig. 6. Knockdown of EZH2 abolished the function of TUG1. (A) Cell MTT assay, (B) colony formation assay, (C) wound healing assay, (D) transwell assay and (E) western blot were performed in PATU 8988 cells transfected with sh-RNA+NC, sh-EZH2+NC, sh-RNA+TUG1 and sh-EZH2+TUG1. All data were represented as the mean $\pm$ S.D. from three independent experiments, * P <0.05, ** P <0.01.

Fig. 7. The expression level of TUG1 was negatively correlated with miR-382 and positively correlated with EZH2 in PC tissues. (A) miR-382 and (B) EZH2 expression levels were negatively (2-tailed Spearman's correlation, $r=-0.421$, $p<0.01$) or positively (2-tailed Spearman's correlation, $r=0.679$, $p<0.01$) correlated with TUG1 expression level in our expression cohort via qPCR.

Discussion

LncRNAs have emerged as key regulators in cellular development and human diseases through modulating gene expression programs at transcriptional, post-transcriptional and

epigenetic levels; for example, Koirala et al. revealed that lncRNA AK023948 is a positive regulator of AKT; Xia et al. demonstrated that lncRNA HOTAIR promotes metastasis of renal cell carcinoma by upregulating histone H3K27 demethylase JMJD3; Xiong H et al. reported that lncRNA HULC triggers autophagy via stabilizing Sirt1 and attenuated the chemosensitivity of HCC cells [16-18]. Targeting lncRNA-based signal pathways might be novel therapy method. There have recently been many studies on PC [19-25]; however, the roles of lncRNAs in PC carcinogenesis are not well understood. Investigating the underlying molecular mechanism by which lncRNAs function in PC might facilitate the exploitation of novel therapeutic targets. Despite many efforts in recent years, the survival rate of PC still remains unsatisfactory. Therefore, investigating the mechanisms underlying the progression of PC might facilitate the development of novel treatments that improve patient prognosis.

LncRNA taurine upregulated gene 1 (TUG1) was initially identified as a transcript upregulated by taurine, as siRNA-based depletion of TUG1 suppresses mouse retinal development [26]. The abnormal expression of TUG1 has been reported in many cancers [27-34]. In the current study, based on the results of lncRNA expression array analysis of clinical PC samples and RT-qPCR, we demonstrated that the lncRNA TUG1 is upregulated in PC and serves as an oncogene in tumorigenesis. Moreover, compared with the normal HPDE6-C cells, TUG1 expression was strikingly higher in four PC cell lines except PATU 8988 cells. Employing loss-of-function and gain-of-function approaches, we identified that TUG1 plays a key role in PC cell proliferation, migration and EMT formation. Deletion of TUG1 significantly weakened PC cell growth ability, decreased cell migration capacity and inhibited the EMT phenotype formation, and vice versa in TUG1 overexpression cells. Our findings revealed that TUG1 may function as an oncogene in PC, and its overexpression facilitated PC development and progression. However, the detailed mechanism by which TUG1 functions in PC warrants further investigation.

Endogenous transcripts containing miRNA response elements (MREs) can co-regulate each other by acting as miRNA sponges or ceRNAs, forming large-scale regulatory networks across the transcriptome [35, 36]. Recently, accumulating lncRNAs have been demonstrated as endogenous decoys for specific miRNAs and regulate their function; for example, in 2017, Sun Y et al. demonstrated that deregulation of miR-183 promotes melanoma development via lncRNA MALAT1 regulation and ITGB1 signal activation; Yang S et al. in 2016, reported that construction of differential miRNA-lncRNA crosstalk networks based on the ceRNA hypothesis uncovered key roles of lncRNAs implicated in esophageal squamous cell carcinoma; and in 2016, Zhao L showed that the lncRNA SNHG5/miR-32 axis regulates gastric cancer cell proliferation and migration by targeting KLF4 [12, 37, 38]. In our study, we provide evidence that TUG1 may function as a ceRNA to competitively sponge miR-382 and influence the distribution on specific targets. Consistent with the function of a miR-382 mimic, deletion of TUG1 could suppress the miR-382 target EZH2, whereas ectopically expressed TUG1 inhibits miR-382 function, leading to suppression of its target gene EZH2. Therefore, the influence of TUG1 on PC cell proliferation, migration and EMT formation could be attributed, at least partially, to function as a ceRNA competitively sponging miR-382.

Conclusion

Generally, we show that TUG1 is upregulated in PC tissues and cell lines. Its effects on cell proliferation, migration and EMT formation indicated its oncogenic property in PC tumorigenesis. In our study, TUG1 acts as a molecular sponge for miR-382 and regulates its target EZH2. This reciprocal repression of TUG1 and miR-382 may highlight the important role of RNA-RNA interaction and provide novel insight into lncRNA-based mechanisms underlying various aspects of tumorigenesis.

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Disclosure Statement

No conflicts of interest to disclose.

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