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Long non-coding RNA TUG1 can promote proliferation and migration of pancreatic cancer via EMT pathway

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Abstract. – OBJECTIVE: This paper aimed to investigate the effect of long non-coding RNA TUG1 (lncRNA TUG1) on cell proliferation, as well as cell migration in pancreatic cancer.

PATIENTS AND METHODS: The mRNA levels of Taurine-up-regulated gene 1 (TUG1) in three kinds of pancreatic cancer cells BxPC3, PaTu8988 and SW1990 was detected by RT-qPCR. Meantime, RT-qPCR was used to examine the mRNA levels of TUG1 in 20 cases of human pancreatic cancer tissues and its para-carcinoma tissues. pCDH-TUG1 plasmid and its empty plasmid pCDH were transfected into BxPC3 and PaTu8988 cells to up-regulate TUG1 expression. siRNA targeting TUG1 and the control siRNA were transfected into SW1990 cells to down-regulate TUG1 expression. Cell clone formation and CCK-8 assay were used to detect the cell proliferation capacity. Transwell assay was used to evaluate cell migration capacity. Western blot was applied to examine the protein expressions of MMP2, MMP9, E-cadherin, Smad 2, Smad 3, p-Smad 2, p-Smad 3, TGF-β and TGF-βR. RT-qPCR was used to detect the levels of MMP2 and MMP9.

RESULTS: The results showed that TUG1 was differentially expressed in the three kinds of pancreatic cancer cells, among which the expression level of SW1990 was relatively high, and the expression levels of BxPC3 and PaTu8988 were relatively low. TUG1 had more expression in pancreatic cancer tissues than that in para-carcinoma tissues. After the up-regulation of TUG1, cell proliferation and migration capacities were increased, protein levels of MMP2 and MMP9 were increased and protein level of E-cadherin was declined. Conversely, after down-regulation of TUG1 expression, cell proliferation and migration capacities were weakened, protein levels of MMP2 and MMP9 were decreased and protein level of E-cadherin was increased. In addition, over-expressed TUG1 could promote Smad2 and Smad3 phosphorylation, but Smad2 and Smad3 phosphorylation were weakened after down-regulated expression of TUG1. The protein expression of TGF-β and TGF-β receptor were more in the TUG1 overexpression group than that in the control group, while the result was just opposite after TUG1 expression was down-regulated.

CONCLUSIONS: These data suggest that lncRNA TUG1 may enhance the proliferation and migration of pancreatic cancer cells through EMT pathway.

Key Words:
lncRNA, TUG1, EMT pathway, Pancreatic cancer.

Introduction

The incidence of pancreatic cancer (PC) is increasing year by year, which has become one of the most common malignant tumors of digestive tract[1]. Pancreatic cancer ranks fourth in the most common causes of cancer death for males and females in the United States. Pancreatic cancer is a kind of cancer with characteristics of high malignant degree, rapid progression, invasion against surrounding tissues and organs in early stage and distant metastasis. Combined with the non-specific symptoms and signs, and the lack of simple and reliable early diagnosis method, it is often in late stage with poor prognosis once diagnosed. Its 5-year survival rate is always lower than 5%, which has not been improved in the past 40 years[2,3]. Therefore, the mechanism of invasion and metastasis of pancreatic cancer in early stage has become a research hotspot.

Long non-coding RNA (lncRNA) is a kind of RNA molecule that does not encode a protein with the length between 200 and 100000 nt. It regulates gene expression at multiple levels and participates in cell growth, differentiation, metabolism and other cell activities[4]. Recent studies have found that lncRNA is abnormally up-regulated or down-regulated in pancreatic cancer, which can promote or inhibit the occurrence and development of tumor; and its role is similar to oncogene or anti-onco-

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gene. As reported, there are various LncRNAs, which are closely related to the pathogenesis of pancreatic cancer, including HOX transcriptional antisense RNA (HOTAIR), metastasis-associated lung adenocarcinoma transcript 1 (MALAT1) and highly up-regulated liver cancer (HULC), etc. The expression of HOTAIR in pancreatic cancer is significantly increased, which can specifically silence the cell cycle factors and interferon, promoting the cancer cell invasion. Knockout of HOTAIR sequence can reduce the proliferation and invasive capacities of cancer cells, and even lead to cancer cell apoptosis[5]. MALAT1 was first found in lung cancer, and it was proved to be increased abnormally in a variety of digestive system tumors, such as liver cancer, colorectal cancer, and pancreatic cancer, in different degrees[6]. In patients with pancreatic cancer, MALAT1 level with lymphatic metastasis is significantly higher than that with negative lymphatic metastasis, and the expression level of MALAT1 is higher in patients with distant metastasis, indicating that MALAT1 has a close relationship with the prognosis of pancreatic cancer. Prensner et al[7] studied and found that the HULC had more expression in pancreatic cancer tissues than that in para-carcinoma tissues, and the HULC expression in pancreatic cancer patient with vascular invasion and lymph node metastasis was significantly higher than that without vascular invasion and lymph node metastasis, so HULC promises to be a new biomarker for predicting the prognosis of patients with pancreatic cancer.

Epithelial-mesenchymal transition (EMT) represents a phenomena that epithelial cells transform into the cells with interstitial phenotype. EMT plays a vital part in various life processes, such as embryonic development, tissue remodeling, chronic inflammation, cancer metastasis and a variety of fibrous diseases. Its main features include less expression of cell adhesion molecules (such as E-cadherin) and more expression of N-cadherin and Vimentin[8,9]. Moreover, there are many EMT markers, such as MMP2, MMP9, Snail and Col-I, that will increase in EMT process. EMT markers often indicate the increase of invasion and metastasis capacities, decrease of differentiation capacity and poor prognosis for various types of tumors[10].

Studies have shown that lncRNA Taurine-up-regulated gene 1 (TUG1) can promote the proliferation of osteosarcoma and bladder cancer cells [11, 12]. However, little has been reported about the expression and biological function of TUG1 in pancreatic cancer cells. In the current study, we aimed to detect the expression of TUG1 in pancreatic cancer and its role in the biological behavior of pancreatic cancer cells, and to investigate its potential molecular mechanism, in order to offer a theoretical evidence for the clinical gene treatment for pancreatic cancer.

Patients and Methods

Main Materials and Reagents

Human pancreatic cancer cell lines SW1990, BxPC3 and PaTu8988 were purchased from ATCC. A total of 30 cases of pancreatic cancer tissue and the corresponding para-carcinoma tissue samples were provided by Beijing Chao-Yang Hospital. This study was approved by the Ethics Committee of Beijing Chao-Yang Hospital. Signed written informed consents were obtained from all participants before the study. TUG1 expression plasmid and the corresponding empty vector pCDH were synthesized by Shanghai GenePharma, China. Dulbecco's Modified Eagle Medium (DMEM) (high glucose) (Hyclone, Logan, UT, USA), fetal bovine serum (Gibco, Grand Island, NY, USA), Lipofectamine™ 2000 (Invitrogen, Carlsbad, CA, USA), TUG1-siRNA and its negative control (Shanghai GenePharma, China), transwell chamber (BD Biosciences, Franklin Lakes, NJ, USA) and CCK-8 kit (Dojindo, Kumamoto, Japan).

RT-qPCR

Total RNA was extracted from each group of cells using Trizol reagent (Invitrogen, Carlsbad, CA, USA). cDNA was synthesized according to instructions of reverse transcription kit, followed by RT-qPCR. Upstream primer sequence of TUG1: 5'-TAG CAG TTC CCC AAT CCT TG-3', downstream primer sequence: 5'-CAC AAA TTC CCA TCA TTC CC-3'; upstream primer sequence of MMP2: 5'-TGA TCT TGA CCA GAA TAC CATC-GA-3', downstream primer sequence: 5'-GGC TTG CGA GGGAAG AAG TT-3'; upstream primer sequence of MMP9: 5'-CCTGGA GAC CTG AGA ACC AAT C-3', downstream primer sequence: 5′-GGC TTG CGA GGG AAG AAG TT-3'; upstream primer sequence of internal control U6: 5'-CTC GCT TCG GCAGCA CA-3', downstream primer sequence: 5'-CCA CCC GAG TGTAAC CAT AGC-3'. Reaction conditions: pre-degeneration at 95°C for 30 s, 95°C for 5 s, annealing at 60°C for 20 s, extension at 72°C for 1 min, a total of 40 cycles. TUG1 primers and internal control U6 were obtained from Guangzhou RIBOBIO, and MMP2 and MMP9

primers were obtained from Sangon Biological Engineering (Shanghai). All data received the relative quantitative analysis using $2^{-\Delta\Delta Ct}$ method. The experiment was repeated three times.

Plasmid Transfection

BxPC3 and PaTu8988 cells in logarithmic growth phase were taken and incubated in the 6-well plate with about 5×10^5 cells per well. When cells grew to 70%-90%, they were transfected with LipofectamineTM 2000 and plasmid at a ratio of 1:2.5 (μg:μL). After transfection for 72 h, the transfection rate and cell morphology were observed and the cells were collected.

siRNA Transfection

SW1990 cells in logarithmic growth phase were taken and incubated in the 6-well plate with about 5×10^5 cells per well. When cells grew to 50%-70%, they were transfected with Lipofectamine™ 2000 and plasmid at a ratio of 1:2.5 (μg:μL). After transfection for 72 h, the transfection rate and cell morphology were observed and the cells were collected.

Clone Formation Assay

Cells in experimental group and control group after transfection for 72 h were taken and inoculated onto the 12-well plates in medium with 0.3% agarose, and three repeating wells were set up in each group. The culture was terminated when visible clone appeared on the 6-well plate. After 2 weeks, colonies were dyed with methyl thiazolyl tetrazolium (MTT – Sigma-Aldrich, St. Louis, MO, USA). Afterwards, a microscope was used to count cells for further measurements. The experiment was repeated for three times.

Transwell Assay

Cells at 48h after transfection were resuspended using serum-free DMEM medium, and the cell density was adjusted to 4×10^5/mL. 100 μL medium was added to the upper chamber and complete culture solution with 20% FBS was applied to the lower chamber. After 24 h, the chamber was taken out and the cells that were not removed inside the chamber were wiped out with medical cotton swab. After washing with phosphate-buffered saline (PBS) for three times, 4% paraformaldehyde solution was added for fixation, followed by staining via crystal violet. Cells were counted in 8 high power fields (200 ×) randomly selected under the optical microscope, and the average was taken. The experiment was repeated three times.

Detection of Cell Proliferation Capacity via CCK-8 Assay

Cells at 48 h after transfection were taken and incubated in the 96-well plate with 2000 cells/well. 5 repeating wells were set up. 10 μL CCK-8 solution was added at 1 d, 2 d, 3 d, 4 d and 5 d, respectively, to incubate cells for 2 h. The absorbance of each well was measured at 450 nm using enzyme-linked immunosorbent detector. The experiment was repeated three times and the average was taken. The growth diagram of curves was drawn with the time as the horizontal axis and relative proliferation rate as the vertical axis.

Western Blot

Cells were harvested after transfection for 48-72 h. Total protein was extracted and a BCA kit (Beyotime, China) was used to detect protein concentration. A total of 30 ug protein was separated via SDS-PAGE gel before transfer to polyvinylidene fluoride (PVDF) membrane. Then PVDF membrane was then blocked for 2 h using 10% none-fat milk at room temperature, and antibodies were added to incubate PVDF membrane overnight. Afterwards, PVDF membrane was put into incubation solution with second antibodies for 1 h at RT, followed by development via ECL chemiluminescence, and analysis via gel imaging system. First antibodies against MMP 2, MMP 9, Smad 2, Smad 3, p-Smad 2, p-Smad 3, TGF-β, TGF-βR and GAPDH were obtained from abcam (Cambridge, MA, USA). Second antibodies against rabbit and mouse were purchased from CST (Cell Signaling Technology, Danvers, MA, USA). The experiment was repeated three times.

Statistical Analysis

SPSS 16.0 software (SPSS Inc., Chicago, IL, USA) was applied for statistical analysis. All experimental data were presented as mean ± standard deviation ($\bar{x}$ ± SD). The *t*-test was used for the comparison of means between two groups. $p<0.05$ suggested a statistical difference.

Results

Expression of TUG1 in Different Pancreatic Cancer Cells and Cancer Tissues and para-Carcinoma Tissues

Three kinds of pancreatic cancer cells, BxPC3, SW1990 and PaTu8988, were selected. RT-qPCR was used to evaluate the mRNA level of TUG1 and its internal control in different cells. As

showed, TUG1 was expressed differently in three kinds of pancreatic cancer cells. The expression level of TUG1 was the highest in SW1990, while that in BxPC3 and PaTu8988 was relatively lower (Figure 1A). Also, RT-qPCR was applied for examining mRNA level of TUG1 in 20 cases of pancreatic cancer tissues and corresponding para-carcinoma tissues. Results showed that the expression of TUG1 in 20 pancreatic cancer tissues was significantly higher compared to that in para-carcinoma tissues (Figure 1B).

Up-Regulation or Down-Regulation of TUG1 Expression

Plasmid transfection: Fluorescent quantitative PCR showed that the relative expression quantities of TUG1 in BxPC3 and PaTu8988 in experimental group were higher compared to those in control group after transfection with TUG1 (p<0.01, Figure 1C).

siRNA transfection: SW1990 was transfected with negative control siRNA, and TUG1 siRNA1, TUG1 siRNA2, and TUG1 siRNA3. The difference of TUG1 siRNA3 was statistically significant (p<0.01). So TUG1 siRNA3 was selected in this study for subsequent experiments (Figure 1D).

Influence of up-Regulation or Down-Regulation of TUG1 on Pancreatic Cancer Cell Proliferation

The results of clone formation assay showed that the clonogenicity of TUG1 up-regulation pancreatic cells was more than that in control group (p<0.01, Figure 2A). In addition, the clonogenicity of TUG1 down-regulation pancreatic cells was less than that in control group (p<0.01, Figure 2B). CCK-8 analysis showed that compared to control group, the cell proliferation capacity of experimental group after up-regulation of TUG1 was increased more significantly with the time; the result was opposite after down-regulation of TUG1 (p<0.01, Figure 2C and 2D). The above results suggested that the up-regulation of TUG1 can enhance cell proliferation in pancreatic cancer, but the down-regulation can inhibit its growth.

Detection of Cell Migration Capacity via Transwell Migration assay

As shown in Figure 3A, the up-regulation of TUG1 increased the migration capacity of BxPC3 and PaTu8988, and the number of migrating cells was more than that of control group significantly (p<0.01); the down-regulation of TUG1 inhibited the migration capacity of SW1990 cells, and the number of migrating cells in control group was significantly increased compared with that in siTUG1 group significantly (p<0.01, Figure 3B).

Influence of TUG1 on Protein Levels in MMP2, MMP9 and E-cadherin in Pancreatic Cancer Cells

As shown in Figure 4A, after TUG1 was over-expressed in BxPC3 and PaTu8988 cells, the expression quantities of MMP2 and MMP9 were higher compared to those in control group, but the expression quantity of E-cadherin was lower compared to that in control group; after TUG1 in SW1990 was down-regulated, the expression quantities of MMP2 and MMP9 in siTUG1 group

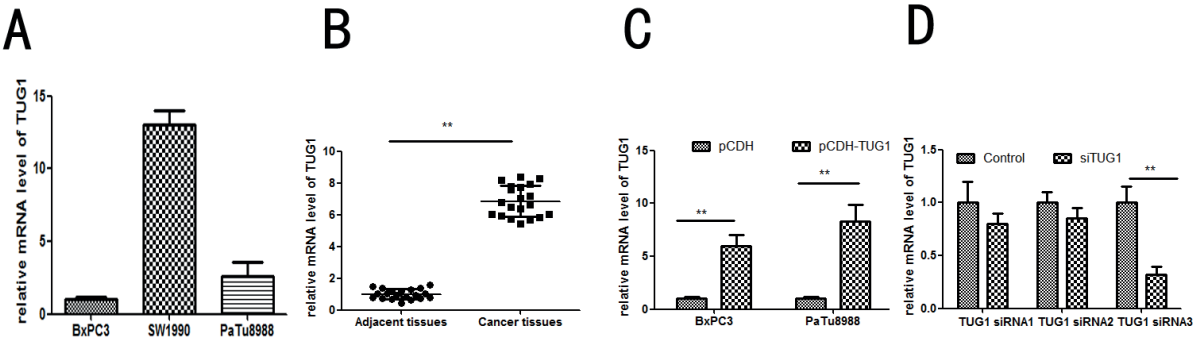

Figure 1. Expression of TUG1 in different pancreatic cancer cells and cancer tissues and para-carcinoma tissues and efficiency of up-regulation or down-regulation of TUG1 expression. ***(A)*** The expression of TUG1 in three human pancreatic cancer cells by RT-qPCR. ***(B)*** The expression of TUG1 in human pancreatic cancer tissues and adjacent tissues by RT-qPCR. **, p < 0.01 for comparisons between two groups. ***(C)*** Efficiency of up-regulation of TUG1 via pCDH-TUG1 plasmid. ***(D)*** Efficiency of down-regulation of TUG1 via TUG1 siRNA. **, p < 0.01 for comparisons between control group and TUG1 siRNA 3 group.

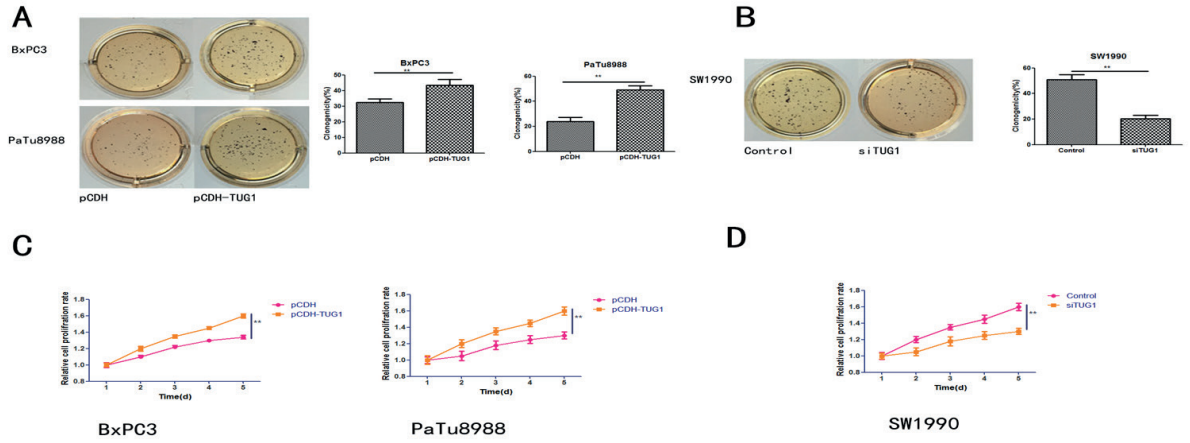

Figure 2. Effects of TUG1 up-regulation and down-regulation on cell proliferation. ***(A)*** The clonogenicity of TUG1 overexpression was detected by colony formation assay in BxPc3 and PaTu8988 cells. ***(B)*** The clonogenicity of TUG1 down-regulation was detected by colony formation assay in SW1990 cells. Colonies were labeled with MTT. The number of colonies was counted. The data are shown as the means ± SD (n = 3). **, $p < 0.01$ for comparisons between groups. ***(C)*** The effects of TUG1 up-regulation and down-regulation on cell proliferation in pancreatic cells detected by CCK-8 assay, **, $p < 0.01$ for comparisons between groups.

were significantly lower than those in control group, but the expression quantity of E-cadherin was significantly higher compared to that in control group. Meanwhile, the mRNA levels of MMP 2 and MMP 9 showed the same trend with the protein levels ($p < 0.01$, Figure 4B and 4C).

TUG1 could regulate the TGF-β/Smad Signaling Pathway

As showed in Western blot analysis (Figure 4D), the protein level of Smad2 and Smad3 were the same regardless of the up-regulation or interference with TUG1 expression compared with those in control group. But the results of phosphorylated Smad2 and Smad3 were different. After TUG1 was up-regulated, the phosphorylation of Smad2 and Smad3 was promoted. But after the expression of TUG1 was decreased, the phosphorylation of Smad2 and Smad3 was decreased. Compared with those in control group, the protein levels of TGF-β and TGF-β receptor protein were increased after the up-regulation of TUG1 expression, but the trend was opposite after the interference with TUG1 expression.

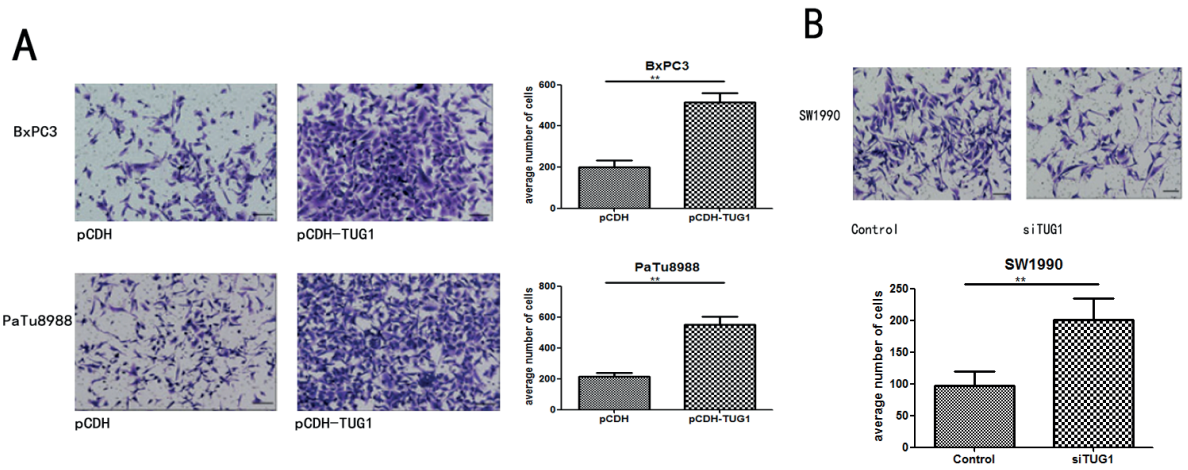

Figure 3. Effects of TUG1 up-regulation and down-regulation on cell migration. ***(A)*** The effects of TUG1 overexpression on cell migration in BxPC3 cells and in PaTu8988 cells detected by transwell assay, **, $p < 0.01$ for comparisons between groups. ***(B)*** The effects of TUG1 down-regulation on cell migration in SW1990 cells detected by transwell assay, **, $p < 0.01$ for comparisons between groups.

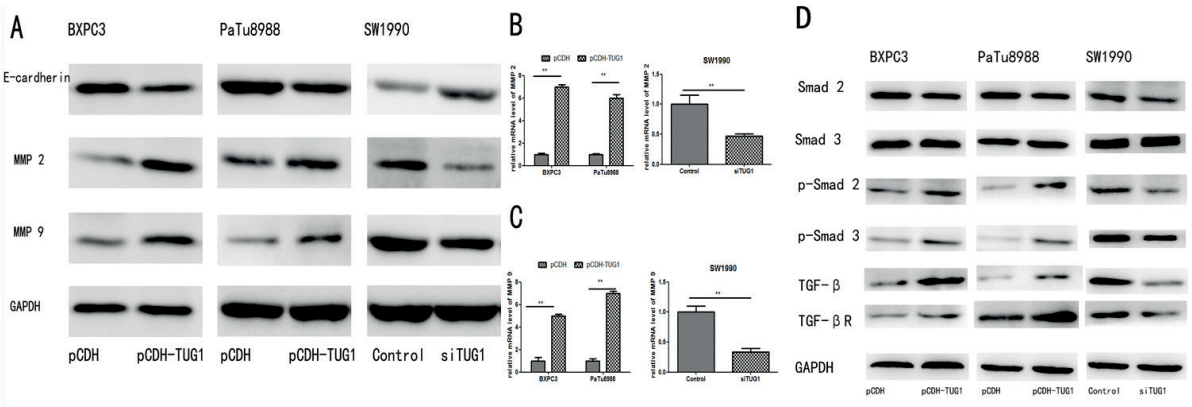

Figure 4. Effect of TUG1 up-regulation and down-regulation on MMP2, MMP9 and E-cadherin expression, as well as the TGF-β/Smad pathway. ***(A)*** The effect of TUG1 up-regulation and down-regulation on the protein levels of E-cardherin, MMP 2 and MMP 9 evaluated by Western blot. GAPDH was loaded as an internal control. (***B*** and ***C***) The effect of TUG1 up-regulation and down-regulation on mRNA levels of MMP 2 and MMP 9 evaluated by RT-qPCR. **, $p < 0.01$ for comparisons between groups. (***D)*** Western blot showed the effect of TUG1 up-regulation and down-regulation on the protein level of Smad 2, Smad 3, p-Smad 2, p-Smad 3, TGF-β and TGF-βR.

Discussion

Pancreatic cancer is one common digestive system cancer with a very high degree of malignancy[13]. The pathogenesis of pancreatic cancer has drawn more attention in recent years, and more and more evidence suggests that the abnormal expression of lncRNA may cause a variety of diseases and dysfunctions, especially tumors[14,15].

TUG1 is the lncRNA with the size of 7.1 kb, and was first discovered in the detection of mouse retinal cells after taurine treatment[16]. Studies have shown that TUG1 can promote the cell proliferation in osteosarcoma, bladder urothelial carcinoma, and other tumors[11,12]. Some studies have also shown that TUG1 expression is down-regulated in non-small cell carcinoma, which is an important regulatory molecule of p53[17]. However, little has been reported about the function and the underlying mechanism of TUG1 in pancreatic cancer.

To determine the effect of TUG1 in pancreatic cancer, we up-regulate TUG1 in BxPC3 and PaTu8988 cells and down-regulate TUG1 in SW1990 cells using plasmid and siRNA in the current study to evaluate the role of TUG1 expression on cell proliferation and migration. Results showed that the up-regulation of TUG1 promoted cell proliferation and migration, but the down-regulation of TUG1 had an opposite effect. To further demonstrate that TUG1 enhanced the migration capacity of pancreatic cancer cells, Western blot analysis was used to examine the content of E-cadherin in transfected pancreatic cancer cells. E-cadherin is the most important member of the calcium-dependent adhesion molecule family. In the cancer tissue, E-cadherin adhesion system is interfered and destroyed via different routes, and its dysfunction often causes the migration of cancer cells[18]. In the current study, results showed that protein expression of E-cadherin was declined after TUG1 was up-regulated, while protein expression of E-cadherin was augmented after TUG1 was down-regulated. This finding further confirms that TUG1 can promote the migration of pancreatic cancer cells. At the same time, the levels of MMP2 and MMP9 in transfected pancreatic cancer cells were detected by Western blot and fluorescent quantitative PCR. Lin et al[19] demonstrated that MMP2 and MMP9 have vital roles in the metastasis of pancreatic cancer, and the up-regulation of MMP2 and MMP9 promotes the metastasis of pancreatic cancer. The results of this study showed that levels of MMP2 and MMP9 were increased after TUG1 was up-regulated, but levels of MMP2 and MMP9 were decreased after interference with TUG1. The above study suggests that TUG1 may have a key role in the migration and proliferation of pancreatic cancer.

Smads protein refers to the related protein involved in TGF-β intracellular signal transduction in different animals and human, which is the intermediary agent that passes the signal from cytoplasm to nucleus, including receptor-regulated, co-regulated and inhibitory Smad protein. Smad2 and Smad3 are receptor-regulated Smad proteins,

and MH1 region at N-terminus, MH2 region at C-terminus and the joining region between them are their common structure. N-terminus has the nucleic acid location sequence motif (NLS) and C-terminus has the serine sequence motif (SSXS). Under unactivated state, MH1 region and MH2 region inhibit each other, and NLS in MH1 region make the Smad and target gene bind in nucleus after activation; MH2 region has the transcriptional activation effect, and its activation can remove the mutual inhibition between MH1 region and MH2 region. Smad4 is the co-regulated Smad protein, which cannot bind to the receptor or be phosphorylated, but can stabilize the structure of Smad oligomer and maintain the effective transcriptional activity of Smad complex.

Transforming growth factor β (TGF-β) family include polypeptide growth factor subfamilies with structural and functional correlation. Smads protein is the important signal transduction and regulatory molecule in TGF-β superfamily cells. Activated receptors can induce the phosphorylation of Smad2 and Smad3 protein, and form complex with Smad4, which can enter the nucleus regulating the transcription of target genes. As the downstream receptor of TGF-β, there are fewer reports on mutations between Smad2 and Smad3, and their expression levels are associated with the prognosis of pancreatic ductal adenocarcinoma. The inactivation of Smad4, as the tumor suppressor gene, is a common change of pancreatic ductal adenocarcinoma, and its incidence is about 50-60%, which is often caused by gene deletion, mutation, and acquired modification. Studies[20-22] have shown that the decreased expression of Smad4 often leads to the poor prognosis of pancreatic ductal adenocarcinoma.

TGF-β/Smad signaling pathway plays a dual role in early stage of human tumor, which can inhibit tumor progression in early stage. On the contrary, TGF-β/Smad signaling pathway promotes the proliferation, migration, and invasion of tumor cells and immune escape in progressive stage. Thus, the functions of TGF-β/Smad signaling pathway are often associated with histocyte and tumor types[23,24]. In pancreatic cancer cells, TGF-β/Smad signaling pathway inhibits the cancer cell proliferation in early stage; when Smad4 is inhibited or even silent, the activation way of TGF-β/Smad signaling pathway is changed, and TGF-β/Smad signaling pathway promotes the cancer cell proliferation, migration, and invasion[25].

In this investigation, it was found that after the over-expression of TUG1, the phosphorylation of Smad2 and Smad3 was increased, the expressions of TGF-β and TGF-β receptor protein were promoted and the expression of Smad4 protein was decreased; but after interference with TUG1 expression, the above trends were just the opposite. Therefore, we conclude that TUG1 can directly regulate the TGF-β/Smad signaling pathway, thus promoting the pancreatic cancer cell proliferation and EMT process.

Conclusions

We found that TUG1 can improve the proliferation and migration capacities of pancreatic cancer cells via EMT pathway, which provides an important way for further research on the mechanism of TUG1 in the development and progression of pancreatic cancer and provides new potential molecular targets to conquer pancreatic cancer.

Found

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Conflict of interest

The authors declare no conflicts of interest.

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