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Original Research Article

LZTS1 promotes proliferation and suppresses apoptosis by inhibiting the activation of AKT/GSK-3β signaling pathway in pancreatic cancer cells

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Abstract

Purpose: *Pancreatic cancer is a kind of harmful human cancer, rated as the seventh leading cause of global death. Research has shown that in various cancers, the expression of Leucine zipper tumor suppressor 1 (LZTS1) has been found low, but its effects on pancreatic cancer is yet to be elucidated. In this research, the aim was to investigate the biological functions of LZTS1 and the underlying molecular mechanism in pancreatic cancer.*
Methods: *GEPIA database was reported for the relative expression of LZTS1 in pancreatic cancer tissues and cell lines compared to normal ones. Kaplan-Meier analysis was done on GEPIA based on the previous data. Gene expression analysis was performed on human pancreatic cancer cell lines (BXPC3, CFPC-1, Panc-1, AsPC-1 and L3.6pl) as well as a normal cell line HEK-293T by employing RT-qPCR. Transfection procedure was done to up- or down-regulate the expressions of LZTS1 in Panc-1 cell line. CCK-8 assay and flow cytometric method were adopted to determine cell viability and apoptosis, respectively. Protein expression levels were determined by Western blot.*
Results: *Expressions of LZTS1 were high in both tumor tissues and cells. Patients with higher LZTS1 had lower 5-year overall survival rate compared to those with lower LZTS1. Overexpressed LZTS1 promoted proliferation and inhibited apoptosis in cancer cell lines. A significant promotion of phosphorylated level of AKT and GSK-3β proteins were achieved through the overexpression of LZTS1.*
Conclusion: *The results from this study revealed that overexpression of LZTS1 increased cell viability and inhibited cell apoptosis, by activation of AKT/GSK-3β signaling pathway. Our findings indicated that LZTS1 might be a prognostic biomarker in pancreatic cancer.*

Keywords: *AKT/GSK-3β, Apoptosis, Cell viability, EMT, LZTS1, Pancreatic cancer*

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INTRODUCTION

In the modern society, pancreatic cancer is considered to be a kind of harmful human cancer, which is the seventh leading cause of global death [1]. The annual death rate of pancreatic cancer in China is nearly equal to the incidence rate [2]. There is about 19.45 % of primarily diagnosed cases in China and 19.27 % all over the world [2]. Although, remarkable progress has been made at the molecular level, yet the prognosis of pancreatic cancer still remains poor. Besides, several evidences proved the existence of termed cancer cells in pancreatic cancer, improved cell progression, drug confrontation, invasion, migration and palindromia [3]. For pancreatic cancer, it has the worst 5-year survival rate (lower than 5 %) in all kinds of hominid cancers and the intermediate value of survival rate is about half a year [4]. With the passage of time, genetic abnormalities have played a significant role in cancer progression [5]. Thus, gene-based treatments have been investigated and there will be more attention about epigenetic modifications of regulating the activity of genes [6-8]. Although, a lot of work has been done, yet the underlying molecular mechanisms are largely unknown.

Leucine zipper putative tumor suppressor 1 (LZTS1) has gradually been unveiled since 1999 [9]. LZTS1 has been found relatively largely expressed in normal tissues but lower or even absent in cancer tissues including bladder, gastric and lung malignant tumors. [10-14]. Moreover, LZTS1 was discovered to be related to down-regulation of tumorigenicity and decrease cell progression at the G2/M stage of cell growth [15]. Former studies have demonstrated that the expressions of LZTS1 are decreased in breast cancer, but only 15 cell lines have been detected [15]. However, the behavior of LZTS1 in pancreatic cancer is still unknown. The present study was aimed at investigating the biological function of LZTS1 and the underlying molecular mechanism in pancreatic cancer.

METHODS

GEPIA database

To get a preliminary knowledge of LZTS1 in pancreatic cancer and normal tissues, GEPIA (http://gepia.cancer-pku.cn/detail.php?gene=LZTS1###) was resorted to and statistical figures based on the previous data provided by Match TCGA normal and GTEx data, concerning the disease PAAD (Pancreatic adenocarcinoma) were formed. The Kaplain-Meier analysis was performed on GEPIA with median as the group cut-off and then the overall survival and disease-free figures were generated accordingly.

Cell culture

Human pancreatic cancer cell lines (BXPC3, CFPC-1, Panc-1, AsPC-1 and L3.6pl) and normal cell line HEK-293T were purchased from American Type Culture Collection (ATCC, Manassas VA, USA). Modified RPMI-1640 medium was used to persevere cells. Meanwhile, cells were put in 10 % fetal bovine serum (FBS) (Thermo Fisher, USA) and then cultured in madid environment with 5 % CO_2 at 37℃.

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

By employing TRIzol reagent (Beyotime, Beijing, China), total RNA extractions were acquired from both cells and tissues. The extracted RNAs were reversely transcribed into complementary DNA (cDNA). Amplification of target genes was conducted using the BeyoFast™ SYBR Green qPCR Mix (Beyotime, Shanghai, China), strictly according to the manufacturer's instructions. GAPDH was used as the internal control. Primer sequences of LZTS1 were: forward, 5'-CCCTGCTCAGCAATTCAGAC-3' and reverse, 5'- CATCTCCTGGGAAAGCCAGA-3'; and the primer sequences of the housekeeping gene GAPDH were: forward, 5'-ACCCAGAAGACTG TGGATGG-3' and reverse, 5'-TCAGCTCAGGG ATGACCTTG-3'. The thermo-cycling conditions employed were: initialization at 95 ˚C for 1 min, followed by 40 cycles of denaturation at 95 ˚C for 15 sec, annealing at 60 ˚C for 15 sec and extension at 70 ˚C for 1 min. Quantification was detected by the $2^{-\Delta\Delta Cq}$ method. The experiment was repeated 3 times and the expression values were presented as mean.

Transfection of cultured cells

In order to overexpress LZTS1, the full-length sequence of LZTS1 was cloned into the overexpression vector pcDNA3.1 (oe-LZTS1) and then inserted into cells (Panc-1) using Lipo293F™ Transfection Reagent (Beyotime, Shanghai, China). Short hairpin RNA was adopted and the shRNA sequencing was designed and synthesized by Genscript for downregulation of LZTS1 to form the sh-LZTS1 group (Shanghai, China). The cells were kept on 12-well plates until 70 % conflux. Empty vector (NC) was used as the negative control group.

CCK-8 assay

In accordance to the manufacturer's protocol, viability was examined via CCK-8 assay (Beyotime, Shanghai, China). Cells from the NC

group, oe-LZTS1 and sh-LZTS1 groups were settled in 96-well plates with 5 × 10^4 cells per well for 24 h after transfection. Then, the viabilities of cells were detected at 0, 24, 48, and 72 h. CCK-8 reagent (20 µl) was supplemented into each well. Besides, the plate was cultured for 1 h at 20 ˚C. Through a microplate reader (Molecular Devices, USA), the well absorbance values were determined at 450 nm wavelength.

Flow cytometric method

Cells from the three groups were harvested and set with 75 % ethanol for 20 min at -4 ℃, 24 h after transfection. Incubated by 20 mg/ml RNase (Sigma), RNA was demoted for 2 h at 37 ºC. To analyze apoptosis, cells were colored with FITCAnnexin V and propidium iodide (Beyotime, Shanghai, China). After this step, cells were analyzed by using Calibur flow cytometry (BD Biosciences, USA) with CellQuest software (BD Biosciences, USA).

Western blot assay

The radioimmunoprecipitation assay (RIPA) lysis buffer was applied to fill cells at 4 °C and cells were cultured for 30 min. Then, all proteins were abstracted, and protein density was measured through Enhanced Bicinchoninic Acid (BCA) Protein Assay Kit (Beyotime, Shanghai, China). Then, 50 µg protein was collected for sample spotting. The protein was separated through 15 % sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) (Beyotime, Beijing, China). The protein was kept on polyvinylidene fluoride (PVDF) membrane (Beyotime, Shanghai, China) within 100 V for 2 h, obstructed with 5 % skim milk powder at 25 ℃ for 1 h, and cultured via primary, anti-p-AKT (1:900; ab38449), and anti-p-GSK-3β (1:800, ab75745) at 4 °C overnight, with GAPDH (1:2000, ab181602) as the internal reference. The protein was first cleaned with PBS, and then affixed using the secondary antibody (1:1000) from Abcam (CA, USA). After electrochemiluminescence development (Bio-Rad Laboratories, Inc.), the band image was gathered through the gel imaging analysis system (Bio-Rad Laboratories, Inc.). The density was demonstrated by rate of the gray value of the target protein band, compared to GAPDH band.

Statistical analysis

All data were presented as mean ± SD of 3 independent replicates. Statistical Package for Social Sciences (SPSS) version 22.0 software (IBM Corp, Armonk, NY, USA) was used to perform a statistical analysis. Data were divided into two groups and compared via T-test. One-way analysis of variance was adopted for comparison and the Least Significant Difference test was the post hoc test. The result differences were statistically significant when p is lower than 0.05.

RESULTS

LZTS1 was up-regulated and correlated with bad prognosis in pancreatic cancer

Through GEPIA analysis based on previous data, differential expression of LZTS1 in various cancers were presented and in pancreatic cancer (PAAD), it had higher expression in the tumor than normal cell line (Fig. 1A). Furthermore, the differential expression in PAAD tumor tissues (n=179) was significantly (P<0.05) higher than that in normal tissues (n=171) (Fig. 1B). In addition, an overall survival analysis was done among 89 patients according to the TCGA data on GEPIA and the results (Fig. 1C) indicated that higher LZTS1 expression level patients showed a lower 5-year survival rate compared to the lower group. Besides, disease-free rate was lower in high LZTS1 group than that in the low group (Fig. 1D). The expression of LZTS1 from RNA level was demonstrated by RT-qPCR in the cell lines (CFPAC-1, BXPC-3, L3.6pl, Panc-1) and the results (Figure 1E) indicated that expression of LZTS1 was up-regulated in cancer cells compared to normal cells.

Up-regulated LZTS1 suppressed cell proliferation and promoted apoptosis in pancreatic cancer

After the pan-1 cell line was transfected with oe-LZTS1 or sh-LZST1, RT-qPCR was used to confirm the expression of LZTS1 after transfection in the two groups compared with the control group. The results (Fig. 2B) of the CCK-8 assay revealed that cell viabilities of Panc-1 cell line were significantly decreased after LZTS1 was downregulated and increased in the oe-LZTS1 group. Flow cytometric apoptosis assays displayed that overexpressed LZTS1 inhibited apoptotic ratio of Panc-1 cells and decrease of LZTS1 in cells, led to the increase in apoptotic rate (Figure 2C).

LZTS1 promoted activation of AKT/GSK-3β signaling pathway *in vitro*

After the analysis of how LZTS1 regulated the relative genes, the phosphorylation protein levels of GSK-3β and AKT inside the selected cell lines were detected by Western blot. Phosphorylation of AKT and GSK-3β were significantly (P<0.05)

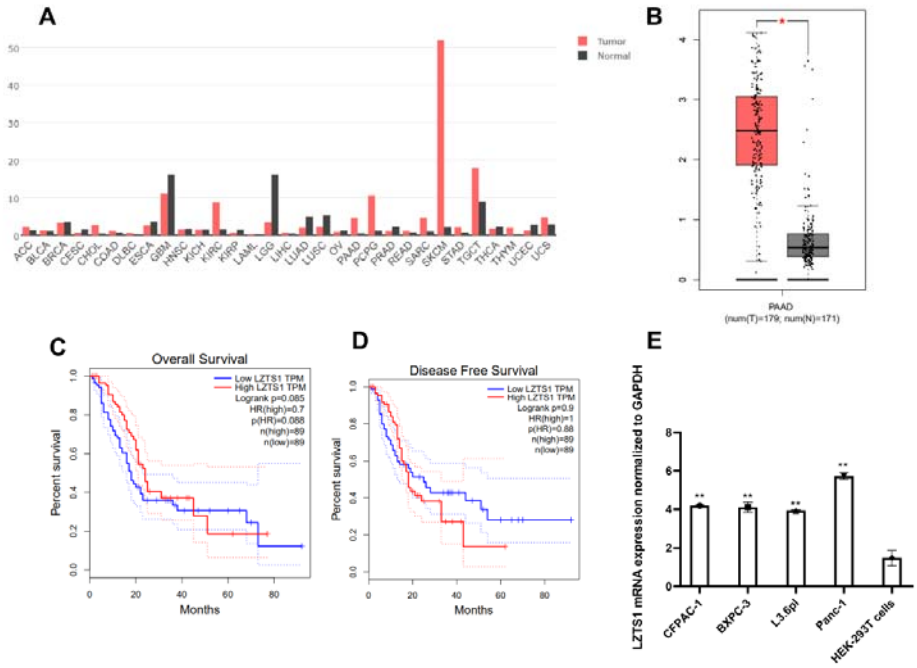

Figure 1: LZTS1 was up-regulated and correlated with bad prognosis in pancreatic cancer. GEPIA database (http://gepia.cancer-pku.cn/detail.php?gene=LZTS1) was used for bioinformatics analysis. (A) Differential expressions in various tumors versus normal tissues. (B) Expressions in PAAD and normal tissues. (C) Overall survival analysis. (D) Disease-free analysis. (E) RT-qPCR assessed the relative expression of LZTS1 mRNA in different PAAD cell lines compared to the normal cell line. All the assays were done three times independently. **: P<0.05.

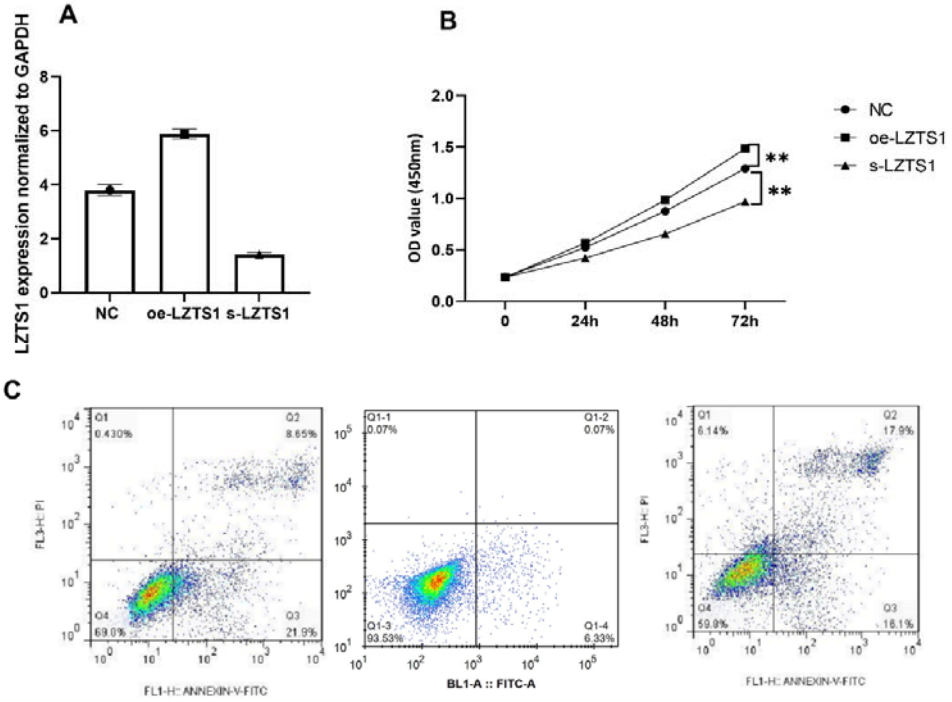

Figure 2: Up-regulated LZTS1 suppressed cell proliferation and promoted apoptosis in pancreatic cancer. Panc-1 cells were selected for the following functional assays after transfection with oe-LZTS1 and sh-LZTS1 compared with the negative control group. (A) RT-qPCR verified the expressions of LZTS1 in the three groups. (B) CCK-8 assay determined cell viabilities at 450 nm. (C) Flow cytometry was used for cellular apoptosis. All the assays were done three times independently. **: P<0.05.

increased in the overexpressed LZTS1 groups and decreased in the sh-LZTS1 group compared with the control groups (Figure 3). The results suggested that overexpressed LZTS1 activated AKT/GSK-3β signaling pathway in Panc-1 cells.

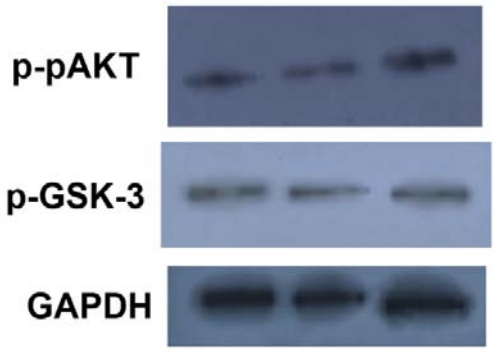

Figure 3: LZTS1 promotes activation of AKT/GSK-3β signaling pathway *in vitro.* Western blotting was used for the examination of p-AKT and GSK-3β with GAPDH as a housekeeping gene

DISCUSSION

The target gene LZTS1 in the research was discovered to be down-regulated in cancers like cutaneous squamous cell carcinoma [16] and osteosarcoma, indicating that it might be an inhibitor in cancers. Decrease in LZTS1 expression was related to a bad prognosis, enhanced cell motility, invasion and EMT of breast cancer [17]. In addition, patients who had pancreatic cancer also showed a higher expression of LZTS1. Thus, we were determined to find the connection between LZTS1 and pancreatic cancer. On GEPIA, tumor tissues showed a higher expression level of LZTS1, as well as cancer cell lines than the normal ones. The Kaplan-Meier plot also indicated that normal patients with high LZTS1 might be shorter, compared to those with lower LZTS1. Nevertheless, there is no report about the relationship between LZTS1 and pancreatic cancer. From this study, it could be proposed that LZTS1 could be an underlying prognosis in pancreatic cancer. Through RT-qPCR, LZTS1 expressed higher in cancer cell lines. As a result of this observation, we investigated the effects of LZTS1 regulation. Compared to the control group, proliferation of cancer cell lines with overexpressed LZTS1 was enhanced while it was reduced in the sh-LZTS1. Cell viability through CCK-8 assay and apoptosis via flow cytometry were examined. The results suggested that overexpressed LZST1 might promote cell viability and inhibit apoptotic rate.

Glycogen synthase kinase 3β (GSK-3β) was previously discovered to adjust cell cycles and proliferation [18]. A few signaling pathways act to inhibit the activity of GSK-3β, leading to nuclear translocation of β-catenin [19]. Loss-of-function of GSK-3β is considered to force cancer progression via accelerating cell cycles and enhancing cell invasion and migration [20]. Activated AKT is known to regulate down-stream protein in relation to cell proliferation, cell cycles [21] and apoptosis [22]. Overexpressed p-AKT is connected with prostate cancer [23]. Therefore, in this study, the relationship between the signaling pathway and pancreatic cancer was discovered. In previous studies, LZTS1 inhibited cell proliferation in colorectal cancer by inhibiting AKT/mTOR [24]. Besides, LZTS1 suppressed hepatocellular cancer cell progression by inactivating the PI3K/AKT pathway [25]. GSK-3β/AKT signaling pathway was determined through Western blot analysis. The LZTS1 activated the phosphorylation of AKT and GSK-3β, which promoted cell growth progression of pancreatic cancer cells. Therefore, LZTS1 may regulate the pancreatic cancer cells growth via the AKT/GSK-3β signaling pathway.

CONCLUSION

The results from this study demonstrated that LZTS1 expression was up-regulated in pancreatic cancer cells, suppressed proliferation and promoted apoptosis. The related AKT/GSK-3β signaling pathway was also activated by overexpression of LZTS1. Our findings suggest that LZTS1 might be an essential biomarker that can be utilized in pancreatic cancer diagnosis.

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DECLARATIONS

Conflict of Interest

There is no conflict of interest attached to this work.

Contribution of the authors

We declare that this work was done by the authors named in this article and all liabilities pertaining to claims relating to the content of this article will be borne by us.

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