ELK3 Mediated by ZEB1 Facilitates the Growth and Metastasis of Pancreatic Carcinoma by Activating the Wnt/B-Catenin Pathway

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Abstract

Background

Rapid progression and metastasis are the major cause of death of pancreatic ductal adenocarcinoma (PDAC) patients. ELK3, a member of ternary complex factor (TCF), has been associated with the initiation and progression of various cancers. However, the role of ELK3 in PDAC need to be further elucidated.

Methods

Online databases and immunohistochemistry were used to analyze ELK3 level in PDAC tissues. The function of ELK3 was confirmed by a series of in vivo and in vitro studies. Western blot and immunofluorescence were used to detect the molecular mechanisms in PDAC. ChIP-qPCR was used to study the mechanism responsible for elevation of ELK3 in PDAC.

Results

ELK3 level was higher in PDAC tissues than that in adjacent normal tissues. Functionally, we demonstrated that ELK3 acted as an oncogene to promote PDAC tumorigenesis and metastasis. Further investigations suggested that ELK3 could promote PDAC cells migration and invasion by activating Wnt/ β -catenin pathway. Then we proved that ZEB1 could directly bind to the promoter of ELK3 to increase its transcription. Finally, both of them were associated with patients' clinicopathological features and worse overall survival.

Conclusions

Our findings enrich the role of ELK3 in PDAC, and provide potential avenues for exploring more effective biomarkers and therapeutic strategies in the treatment of PDAC.

Introduction

Pancreatic ductal adenocarcinoma (PDAC) remains an intractable disease with a 5-year survival rate of 9%, which is the lowest among the different types of cancer[1]. Due to lack of effective screening tool for early detection, most patients are diagnosed with metastatic or advanced tumor, while vast majority of patients relapse within one year, even for early stage of disease after surgical resection [2]. Rapid progression and distant metastasis are the leading cause of poor prognosis for PDAC patients [3, 4]. Therefore, it is paramount to identify the underlying molecular pathophysiology and effective therapeutic targets.

ELK3 is a member of ternary complex factor (TCF), one subfamily of ETS domain-transcription factors [5]. The function of ELK3 is capable of forming a ternary complex with serum response factor (SRF) to regulate gene expression [6, 7]. Several studies have demonstrated that ELK3 is associated with the initiation and progression of various cancers. For instance, ELK3 suppression results in extensive

changes in breast cancer cell genome expression profiles, thus decreases cell migration and metastasis during tumor progression [8]. In squamous cell carcinoma (SCC), ELK3 knockdown severely impairs tumor growth and prohibits progression from benign papillomas to SCC [9]. In addition, ELK3 has also been proved to be associated with chemoresistance [10], angiogenesis and wound closure [11]. Although increasing evidence has demonstrated the biological importance of ELK3, the role of it in PDAC and the potential molecular mechanisms have not been fully elucidated.

Epithelial-mesenchymal transition (EMT) has been considered an essential biological process associated with tumor metastasis, wherein epithelial cells alter their shape, modify the adhesion molecules, and acquire migratory and invasive behaviors [12–15]. EMT process is driven by multiple transcription factors, notably Twist, Zeb1, bHLH, Snail and Slug, and these factors orchestrate and coordinate to repress epithelial marker E-cadherin and activate the expression of mesenchymal markers such as N-cadherin and Vimentin [16, 17]. The reprogramming of these genes expression are initiated and controlled by multiple signaling pathways, and among these, Wnt/ β -catenin plays a critical role in the induction of EMT [18, 19]. Once the canonical Wnt pathway is activated, β -catenin translocates to the nucleus, where it complexes with T cell factor and lymphoid enhancer binding factor (TCF-LEF) to activate the transcription of genes that favour EMT [20–22]. Studies have shown that approximately 80% of colorectal carcinoma has nuclear accumulation of β -catenin, which is associated with poor prognosis [23, 24]. Consistently, hyperactivation of the Wnt/ β -catenin signaling pathway increases susceptibility to hepatocellular carcinoma development [25]. However, the role of the Wnt/ β -catenin pathway in PDAC is less distinct and somewhat controversial [26]. Hence, it is necessary to explore the molecular mechanisms of Wnt/ β -catenin signaling in PDAC.

In the present study, we identified the upregulation of ELK3 in pancreatic cancer tissues firstly. Then we demonstrated ELK3 to be an oncogenic protein that accelerated PDAC cells proliferation and invasion through activating β -catenin signaling pathway. Furthermore, we proved that ELK3 was regulated by ZEB1, which could bind to the promoter of ELK3 and activate its expression. In clinic, ZEB1 was also upregulated in PDAC, and both of them were related to tumor progression and poor survival in PDAC patients.

Results

ELK3 is highly expressed in PDAC

To clarify the role of ELK3 in PDAC, we first analyzed the expression of ELK3 using Oncomine database. The mRNA level of ELK3 was significantly upregulated in pancreatic cancer tissues in two datasets (Badea Pancreas Statistics, $P = 6.36E-9$, Segara Pancreas Statistics, $P = 8.46E-5$) (Fig. 1A). Furthermore, overexpression of ELK3 in PDAC was confirmed by analyzing two NCBI Gene Expression Omnibus (GEO) datasets (GSE15471, $P = 4.78E-10$, GSE71987, $P = 1.08E-7$) (Fig. 1B) and The Cancer Genome Atlas (TCGA) dataset ($P < 0.05$) (Fig. 1C). The Kaplan-Meier curve analyses revealed that elevated ELK3 level indicated poorer overall survival (OS) and relapse free survival (RFS) ($P < 0.05$) (Fig. 1D). To further

determine the expression level of ELK3, immunohistochemical (IHC) analysis of the tissue microarray (TMA) containing 70 cases of PDAC tissues with corresponding normal tissues were conducted. As shown in Fig.1E and F, the expression level of ELK3 was higher in PDAC tissues than that in adjacent normal tissues. Overall, we conclude that ELK3 is frequently elevated in PDAC, and the roles of ELK3 remain to be explored.

ELK3 promotes PDAC cells proliferation, migration and invasion in vitro

To investigate the functional roles of ELK3 in PDAC, ELK3 was knocked down and overexpressed in PANC-1 and MIA PaCa-2 cells, which have been used in our previous experiments [27]. We constructed three shRNAs (sh-1, sh-2, sh-3) and a lentiviral overexpression vector targeting ELK3. The qRT-PCR results showed that ELK3 level was significantly down-regulated or up-regulated in PDAC cells transfected with the indicated shRNAs or overexpression vector respectively (Fig. S1A). Among the three shRNAs, sh-1 (sh-ELK3) was selected for further study because of its highest inhibitory efficiency. In addition, the successful knockdown and overexpression of ELK3 were confirmed at protein levels (Fig. S1B). Colony formation and EdU assays revealed that knockdown of ELK3 suppressed the proliferation of both PANC-1 and MIA PaCa-2 cells (Fig. 2A-B). Conversely, overexpression of ELK3 had the opposite effects on cell proliferation (Fig. 2A-B). The wound-healing assay demonstrated that ELK3 depletion inhibited the mobility of PANC-1 cells, while forced expression of ELK3 increased the migration speed of them (Fig. 2C). Correspondingly, the effect was confirmed by transwell migration and matrigel invasion assays (Fig.2D). Additionally, similar results were obtained in MIA PaCa-2 cells (Fig. 2C-D). To conclude, our findings indicate that ELK3 may be involved in cell proliferation, migration and invasion, acting as a positive regular.

ELK3 promotes pancreatic tumor growth and metastasis in vivo

To verify the function of ELK3 in pancreatic tumor growth and metastasis in vivo, we injected pancreatic cancer cells with stable knockdown or overexpression of ELK3 into the armpit or tail vein of nude mice. The results showed that tumors in sh-ELK3/MIA PaCa-2 group grown more slowly than those in sh-NC/MIA PaCa-2 group, and this phenomenon was also reflected by tumor volume and final tumor weight (Fig. 3A-C). Additionally, the volume and weight of xenograft tumors in ELK3/PANC-1 group were significantly higher than the control tumors of PANC-1 cells (Fig. 3D-F). In the lung metastasis model, we discovered that the metastatic nodules in mice injected with sh-ELK3/ MIA PaCa-2 cells were less than in mice injected with sh-NC/ MIA PaCa-2 cells (Fig. 3G-H), while a higher number of metastatic nodules was observed in mice injected with ELK3/PANC-1 cells than in these injected with Ctrl/PANC-1 cells (Fig. 3I-J). Taken together, these in vivo results suggest that ELK3 plays an important role in pancreatic tumor growth and metastasis.

ELK3 is required in TGF β -induced EMT

As we all know, EMT process plays important roles in cancer cell invasion and tumor metastasis [16]. TGF β is a potent inducer of EMT, and TGF β stimulation could irritate changes in cell morphology and

biological behavior [28, 29]. Our results have showed that ELK3 was associated with malignant progression of pancreatic cancer. This prompted us to explore the underlying effects of ELK3 on TGF β -induced EMT process. Western blot and confocal immunofluorescence analysis indicated that TGF β treatment markedly decreased E-cadherin and increased N-cadherin and Vimentin expression (Fig. 4A-B). However, in sh-ELK3/PANC-1 and sh-ELK3/MIA PaCa-2 cells simultaneous treatment with TGF β , these molecular events induced by TGF β were completely abolished by ELK3 depletion (Fig. 4A-B). Additionally, ELK3 knockdown also effectively quenched the wound healing, cell migration and invasion abilities induced by TGF β in PANC-1 and MIA PaCa-2 cells (Fig. 4C-D). These results demonstrate that ELK3 is crucial for TGF β -induced EMT in PDAC.

ELK3 promotes the progression of pancreatic cancer cells through Wnt/ β -catenin signaling pathway

Considering the importance of β -catenin signaling in the development of cancer and EMT process [30, 31], we were inspired to explore whether ELK3 could regulate Wnt/ β -catenin signaling pathway in pancreatic cancer. To verify the effects of ELK3 on Wnt/ β -catenin, we performed western blot assay. The results showed that neither knockdown nor overexpression of ELK3 significantly affected the total β -catenin level (Fig. 5A). However, ELK3 depletion decreased the level of nuclear β -catenin and increased the cytosolic β -catenin levels, whereas ELK3 overexpression had the opposite effects on the subcellular location of β -catenin (Fig. 5A). TOP-Flash and FOP-Flash luciferase reporters were used to further test the activity of the Wnt/ β -catenin signaling pathway. As shown in Fig. S2, the TOP/FOP luciferase activities in PDAC cells transfected with sh-ELK3 groups were much lower than in the control group, and were significantly higher in ELK3 overexpressed cells. Furthermore, to determine whether β -catenin is essential for the functions of ELK3, ELK3/PANC-1 and ELK3/MIA PaCa-2 cells were transfected with si β -catenin. We observed that β -catenin suppression dampened ELK3-mediated cell wound healing (Fig. 5B), migration and invasion (Fig. 5C). These data confirmed Wnt/ β -catenin signaling pathway plays a vital role in ELK3-mediated pancreatic cancer progression.

ZEB1 transcriptionally activates ELK3 expression

To dissect the molecular mechanism of ELK3 overexpression in PDAC, we first explored the genetic or epigenetic dysregulation of ELK3 in pancreatic adenocarcinoma (TCGA, Firehose Legacy) from the cBioPortal database. However, we discovered no evidence regarding the dysregulation of ELK3 at the genetic or methylation levels (Fig. S3A), suggesting that genetic alterations (mutation, amplification and deletion) and methylation modification may not be the main causes of the overexpression of ELK3 in PDAC. Then, we would explore its overexpression at transcriptional level. As one of the most important EMT-inducing transcription factors, ZEB1 not only transcriptionally represses but also activates some EMT-related genes, and its overexpression promotes tumorigenesis and metastasis in human carcinomas [32-34]. A recent study showed that ZEB1 could collaborate with ELK3 to regulate gene expression [35]. Thus, we are inspired to explore whether ZEB1 could transcriptionally activate ELK3 expression in PDAC. Analyzing from the JASPAR database, we found the binding motifs of ZEB1 and five potential ZEB1 binding sites on the ELK3 promoter (Fig. 6A, B and Fig. S3B). QRT-PCR and western blotting analysis

demonstrated that forced expression of ZEB1 significantly increased ELK3 mRNA and protein levels, while ZEB1 deletion exhibited an opposite effect (Fig. 6C, D and Fig. S3C, D). ChIP-qPCR results indicated that ZEB1 could interact with ELK3 promoter within the -641 to -631bp region (Fig. 6E-F). In addition, we found a significantly decreased ZEB1 enrichment in the ELK3 promoter following ZEB1 silencing, while ZEB1 overexpression increased occupancy of ZEB1 in the ELK3 promoter (Fig. 6G). To further investigate the regulatory role of ZEB1 on ELK3 transcription, we constructed wild type (WT) and mutant (Mut) reporter plasmids. For mutant plasmid, several bases were replaced in the binding site#2, and wild type reporter contained intact binding site#2 (Fig. 6H). Luciferase reporter assays showed that overexpression of ZEB1 could activate the luciferase activity of WT plasmids, but failed to activate Mut reporters (Fig. 6I). To sum up, we concluded that ZEB1 binds to the region between -641 to -631 bp of the ELK3 promoter to activate its' transcriptional activity in pancreatic cancer.

ELK3 is critical for the function of ZEB1 on PDAC cell proliferation and migration

ZEB1 has been shown to promote the proliferation and metastasis of PDAC cells [34]. Since ZEB1 could increase ELK3 level in PDAC, we investigated whether ELK3 was necessary for mediating the effect of ZEB1 on the cellular proliferation and metastasis of PDAC. As shown in Fig. 7A and Fig. S4A, ZEB1-enhanced cell proliferation was inhibited by ELK3 knockdown. Moreover, ZEB1-enhanced cell migration and invasion ability was decreased when ELK3 was knockdown (Fig. 7B, C and Fig. S4B, C). As an EMT-activator, western blot and immunofluorescence results shown that ZEB1 could promote the EMT process of PDAC cells, while this effect was reversed by ELK3 knockdown (Fig. 7D, E, and Fig. S4D, E). In summary, these results demonstrated that ELK3 was important for the oncogenic effect of ZEB1 on PDAC progression.

Clinical pathological features of ZEB1 and ELK3 in PDAC patients

First, we found that ZEB1 expression was also upregulated in GSE15471 ($P = 1.75E-07$) and GSE71987 ($P = 6.79E-06$) datasets (Fig. S5A). Scatter plot analysis showed a positive correlation between the mRNA levels of ZEB1 and ELK3 (GSE15471, $R = 0.6792$, $P < 0.0001$, GSE71987, $R = 0.8890$, $P < 0.0001$) (Fig. S5B). In addition, the protein level of ZEB1 was examined in above 70 paired pancreatic cancer tissues and paracancerous tissues, and the results of IHC analysis showed that ZEB1 was highly expressed in pancreatic cancer tissues compared with matched normal tissues (Fig. 8A-B). Moreover, nearly 64.3% of pancreatic cancer samples where ZEB1 was more highly expressed presented stronger ELK3 staining, while approximately 71.2% of those with lower ZEB1 expression exhibited weaker ELK3 staining (Fig. 8C). Pearson correlation analysis confirmed the positive correlation between ZEB1 and ELK3 proteins in TMAs ($R = 0.848$, $P < 0.0001$) (Fig. S5C). Based on the median expression of ELK3 or ZEB1 in TMAs, the samples were divided into ELK3 high expression group and ELK3 low expression group or ZEB1 high expression group and ZEB1 low expression group. As shown in Table1 and Fig. S5D, ELK3 expression was significantly higher in pancreatic cancer tissues of T3 stage, N1stage, distant metastasis M1 stage and AJCC stage IIB-IV than in these of T1-T2 stage, N0 stage, M1 stage and AJCC-IIA stage respectively ($P < 0.05$ for all). ZEB1 expression was positive with pathological grade, N stage and AJCC stage ($P <$

0.05 for all, Table2, Fig. S5E). Kaplan-Meier survival analysis showed that patients with higher ELK3 and ZEB1 expression level were both associated with worse overall survival (OS) (Fig. 8D,E). Moreover, the combination of these two elements demonstrated that pancreatic cancer individuals with the expression of ZEB1^{high}ELK3^{high} had an even worse OS rate than any other groups (P = 0.0014) (Fig. 8F). Taken together, ZEB1 and ELK3 predicated poor survival in clinical samples and might be indicators of efficient prognostic factors in PDAC patients.

Discussion

The novel ZEB1-ELK3 axis is the crucial finding in the present report. In our study, ELK3 could accelerate PDAC cells proliferation, migration, invasion and EMT process in vitro and promote tumor growth and metastasis in vivo. Mechanistic investigations suggested that ELK3 could activate β -catenin signaling pathway thereby promoting migration and invasion of PDAC cells. Following investigations revealed that ZEB1 regulated the expression of ELK3 in PDAC. Meanwhile, both of them were overexpressed in pancreatic cancer tissues and high ZEB1 and ELK3 expression were both closely associated with patients' clinicopathological features and worse overall survival, indicating ZEB1 and ELK3 may be efficient diagnostic and therapeutic targets in PDAC.

ELK3 is a transcriptional repressor in the subfamily of ETS domain-transcription factors and it contains two special inhibitory domains, the NID and CID [36], and it switches to a transcriptional activator in response to activation of the MAPK/ ERK1/2 pathway [37]. Numerous researches have reported ELK3 played important roles in various physiological processes. In breast cancer cells, ELK3 promoted the cell migration and invasion by providing oncogenic miRNAs through exosomes [38]. In hypoxic condition, ELK3 is downregulated and participates in the regulation of HIF-1 α protein stability. Meantime, ELK3 also regulates hypoxic induction of genes in response to hypoxia [39]. Importantly, ELK3 is required for angiogenesis, and ELK3 mutant mice exhibit decreased rate of wound closure [40]. Given the importance of ELK3 in these processes, we firstly examined the expression of ELK3 in GEO and TCGA databases, and found ELK3 was overexpressed in pancreatic cancer tissues compared with adjacent normal tissues. Then IHC analysis of the TMA confirmed the overexpression of ELK3 in PDAC tumor tissues. Functionally, based on the stable ELK3 knockdown and forced expression cells, we discovered that ELK3 facilitated PDAC cells proliferation, migration and metastasis in vitro and in vivo. The aberrant activation of EMT program has been proved to play an important role in the mechanisms of cancer cell migration and invasion [41]. In our present study, we found that ELK3 depletion can not only significantly attenuate TGF β -induced molecular events, but also inhibit TGF β -induced cell wound healing, migration and invasion abilities. These data suggest that ELK3 acts as a tumor oncogene to foster pancreatic cancer progression, indicating ELK3 has a potential to be a therapeutic targets for pancreatic cancer.

Wnt/ β -catenin pathway, known as the canonical Wnt pathway, is overactivated in multiple cancer types, which is considered a crucial signaling pathway to accelerate the EMT process [18]. Under normal circumstances, E-cadherin and β -Catenin can form a complex located at cell-cell adherent junctions in the membrane. TGF β or EGF stimulation may activate EMT though disassociation of this complex to release

β -catenin, which then translocates into the nucleus to regulate genes expression [42]. Thus, β -catenin may act as a vital regulator in EMT. Our study showed that ELK3 knockdown decreased the level of nuclear β -catenin, while ELK3 overexpression increased the accumulation of β -catenin in the nucleus. The results suggest that ELK3 may promote pancreatic cancer procession through activating the Wnt/ β -catenin pathway. To further verify this hypothesis, a series of rescued experiments were conducted. ELK3 overexpressed PDAC cells were transfected with si β -catenin, and the results proved that β -catenin suppression could reverse ELK3-mediated cell mobility. Collectively, our data demonstrated the effects of ELK3 on β -catenin signaling indicated the important roles of ELK3 in pancreatic cancer progression and EMT process. However, the specific mechanisms of how ELK3 regulates β -catenin pathway need further investigation.

ZEB1, as the core EMT inducer, is a pivotal factor in the tumorigenesis, invasion and metastasis in pancreatic cancer [34]. ZEB1 can regulate the expression of its target genes by recruiting corepressors or coactivators. For example, ZEB1 form complex with NuRD to contribute to the degradation of E-cadherin, thus promoting metastasis in lung cancer [43]. In addition, ZEB1 could recruit deacetylase HDAC1 and HDAC2 to attach to the E-cadherin promoter, resulting in histone deacetylation and reduction of E-cadherin expression in PDAC [44]. Our study verify the importance of ELK3 in the EMT process, which makes it feasible to speculate whether there is a link between ELK3 and ZEB1 during this process. Here, ZEB1 was proved to be highly expressed in PDAC and positively correlated with ELK3. Further molecular experiments demonstrated that ZEB1 could bind to ELK3 promoter and transcriptionally activate ELK3 expression. However, it is necessary to make a further exploration to identify whether ZEB1 can combine with ELK3, the specific mechanism of their combination and how to regulate gene expression after their combination. Taken into account the importance of ZEB1 and ELK3 in EMT process and metastasis, we finally analyzed the correlation of ZEB1 and ELK3 expression with clinical pathological characteristics and prognosis of PDAC patients. We found that both high ZEB1 and ELK3 expression indicated poor prognosis.

Conclusions

In summary, our study illustrated the oncogenic role of ELK3 in pancreatic cancer cell proliferation, migration and invasion. Overexpression of ELK3 promotes the EMT process and activates the β -catenin signaling pathway. In addition, ZEB1 up-regulation contributes to the abnormal expression of ELK3. Our findings enrich the role of ELK3 in PDAC, and provide potential avenues for exploring more effective biomarkers and therapeutic strategies in the treatment of PDAC.

Materials And Methods

Cell lines

The human pancreatic cancer cell lines PANC-1 and MIA PaCa-2 were obtained from Type Culture Collection of the Chinese Academy of Science (Shanghai, China). Both of them were cultured in DMEM

(Gibco) supplemented with 10% fetal bovine serum (FBS; Gibco) and 1% penicillin-streptomycin at 37°C with 5% CO₂.

Tissue microarray (TMA)

The clinical characteristics of ELK3 and ZEB1 expression in PDAC patients were analyzed using TMAs containing 70 pairs of pancreatic cancer samples, which were purchased from Shanghai Outdo Biotech (Shanghai, China). The TMAs contains patients' complete clinicopathological information and follow-up data.

Immunohistochemistry (IHC)

After deparaffinated and dehydrated, the TMAs were boiled in sodium citrate solution (0.01 M, pH 6.0) for 15 min. Then 3% hydrogen peroxide was used to block endogenous peroxidase activity. Next, the TMAs were incubated with ELK3 antibody (1:200, Sigma) and ZEB1 antibody (1:200, Sigma) respectively at 4°C overnight. Next day, the TMAs were incubated with secondary antibody for 1 h at room temperature. IHC staining scores were based on staining intensity and percentage of positively stained cells. The staining intensity was divided into four levels, 0 (negative staining), 1 (weak staining), 2 (moderate staining) and 3 (strong staining). Percentage of positively stained cells was scored as 0 (0–10%), 1 (10%-25%), 2 (25%-50%), 3 (50%-75%) and 4 (75%-100%). The final score was acquired by multiplying the above two scores. Total score of ≤ 4 indicated low expression and > 4 indicated high expression. The score was assessed by three proficient pathologists independently.

Construction of stable knockdown and overexpressed cell lines

Stable knockdown and overexpression of ELK3 were achieved by construction of lentivirus vector (OBiO Biotechnology, Shanghai, China). PANC-1 and MIA PaCa-2 cells were cultured in 6-well plates. When the cells reached 60%-70% confluence, they were infected with appropriate lentiviruses in the presence of 6 µg/ml polybrene (Hanbio Biotechnology, Shanghai, China). Infected cells were selected using 4 µg/ml puromycin (Sigma, USA) for 2 weeks. The transfection efficiency was determined by qRT-PCR and western blotting analysis.

Plasmid construction, RNAi and cell transfection

The overexpression vector and small interfering RNAs (siRNAs) specifically targeting genes were synthesized by RiboBio (Guangzhou, China). Transfection of plasmid or siRNAs in pancreatic cancer cells were performed using Lipofectamine™2000 (Invitrogen, USA) following the manufacturer's instructions. The transfection efficiency was determined by qRT-PCR and western blotting analysis.

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

Total RNA was isolated from cells using RNAiso Plus Reagent (TakaRa) and was reverse-transcribed to cDNA using PrimeScript™ RT reagent kit (TakaRa). Then, SYBR® Premix Ex Taq™ (TakaRa) was used to

amplify cDNA. Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was used as the internal control to normalize the data. The relative gene expression of mRNAs was determined by the $2^{-\Delta\Delta C_t}$ method.

Western blotting analysis

Pancreatic cancer cells were lysed in RIPA buffer containing protease and phosphatase inhibitors. BCA Protein Assay Kit (Beyotime Biotechnology, China) was used to measure the concentration of protein. Protein samples were separated by SDS-PAGE at 90 V for 2 h and transfected to PVDF membranes for 2 h. After blocked in 5% fat-free milk for 1.5 h, the membranes were incubated with primary antibodies at 4°C overnight. Next day, the membranes were incubated with secondary antibodies for 2 h. Finally, they were washed using TBST and detected by ECL chemiluminescent reagent (Millipore, USA).

Cell wound healing assay

Pancreatic cancer cells were cultured in 6-well plates. When the cells grown to full confluence, a scratch wound was made in the center of the well using a 200 μ l plastic micropipette tip. Wound healing images were captured at 0 h and 24 h after injury. The width of wound healing was quantified and compared with baseline values.

Cell migration and invasion assays

Cancer cells of $2-5 \times 10^4$ in serum-free were seeded into upper chamber of the 24-well plates (Corning, USA) with an 8.0 μ m pore size polycarbonate membrane without (migration) or with (invasion) matrigel. The medium in the bottom chamber contained with 10% FBS as the chemoattractant. After 24 h, the cells in the upper chamber were removed using a cotton swab. The cells that migrated and invaded to lower chamber were stained with 0.1% crystal violet and counted in five random fields under the microscope.

Confocal immunofluorescence assay

When cells seeded in the confocal plates grown into 50%-60% confluence, they were washed with PBS, fixed in 4% paraformaldehyde for 20 min, permeabilized with 0.1% Triton X-100 for 20 min and blocked with 5% goat serum for 1 h at room temperature. Then the cells were incubated with primary antibodies at 4°C overnight followed by incubation with fluorophore-conjugated secondary antibody (Cell Signaling Technology) for 1 h. Following washing, the samples were stained with DAPI and imaged using a confocal microscope (Leica, Germany). The primary antibodies were listed as follows: E-cadherin (1:100, Cell Signaling Technology), N-cadherin (1:100, Cell Signaling Technology), Vimentin (1:250, Abcam) and β -catenin (1:250, Abcam).

Chromatin immunoprecipitation (ChIP) assay

ChIP experiments were performed using 1×10^7 pancreatic cancer cells with the ChIP kit (Cell Signaling Technology) according to the manufacturer's protocol. Briefly, PDAC cells were cross-linked with 1% formaldehyde. Then the cells were lysed and chromatin was harvested and fragmented using enzymatic digestion. The chromatin was subjected to immunoprecipitation using ChIP-grade antibody against ZEB1 (Sigma, USA). After immunoprecipitation, the protein-DNA cross-links were reversed and the DNA was

purified, followed by qRT-PCR. The enrichment percentage = $2\% \times 2^{(C[T]_{\text{Input Sample}} - C[T]_{\text{IP Sample}})}$. The qRT-PCR products were used for DNA electrophoresis and visualized by ethidium bromide staining.

Luciferase reporter assay

The luciferase reporter plasmids (pGL3-Luc containing ELK3 promoter, pGL3-Luc containing intact binding site#2 in the ELK3 promoter and pGL3-Luc containing mutant binding site#2 in the ELK3 promoter) were synthesized by GenePharma (Shanghai, China). PDAC cells were co-transfected with approximately 80 ng Luciferase reporter plasmids and 100 ng si-ZEB1 or ZEB1 overexpression vector using Lipofectamine™2000 (Invitrogen, USA). After incubation for 36 h, luciferase activities were detected using the Dual Luciferase Reporter Assay System (Promega, USA) and calculated with the ratio of firefly luciferase/renilla luciferase activity.

Animal experiments

To study primary tumor growth in vivo, 4-week-old male BALB/c nude mice were chosen and maintained in specific pathogen-free conditions. The mice were randomly divided into 4 groups (n = 5), stable cells lines (1.0×10^7) were subcutaneously injected into the armpit of the nude mice. Tumor volumes were estimated in accordance with the formula $(\text{length} \times \text{width}^2)/2$ and measured twice a week. After 21 days, the mice were sacrificed and subcutaneous tumors were removed and weighed. For in vivo lung metastasis model, the nude mice were randomly divided into 4 groups (n = 5), and 2×10^6 cells were injected through the tail vein. Six weeks later, all the mice were killed, and the lungs were removed, imaged, paraffin-embedded and stained with hematoxylin and eosin (H&E).

Statistical analysis

SPSS 22.0 was conducted for statistical analysis. Significant correlations between ELK3/ZEB1 expression and clinicopathological features of PDAC patients were analyzed by Student's t-test or the Mann-Whitney U test and Pearson χ^2 test. Kaplan-Meier method and a log-rank test were used to evaluate survival difference. $P < 0.05$ was considered statistically significant for all tests.

Abbreviations

PDAC: Pancreatic ductal adenocarcinoma

TCF: Ternary complex factor

EMT: Epithelial-mesenchymal transition

TMA: Tissue microarray

qRT-PCR: Quantitative real-time PCR

ChIP: Chromatin immunoprecipitation

IHC: Immunohistochemistry

H&E: Hematoxylin and eosin

siRNA: Small interfering RNA

shRNA: Short hairpin RNA

NC: Negative control

Ctrl: Control

Declarations

Ethics approval and consent to participate

The application of TMAs complied with relevant regulations, and present study was approved by the Ethics Committee of Shanghai General Hospital. The animal experiments in this study were approved by the Animal Care Committee of Shanghai General Hospital.

Consent for publication

All participants were consent for publication.

Availability of data and materials

The raw data of the results and materials in this article will be made accessible by the authors, without undue reservation.

Competing interests

The authors declare that they have no conflicts of interest.

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Authors' contributions

ZQY designed experiments, performed experiments, analyzed data, prepared figures, and wrote the manuscript, RYC analyzed data and designed experiments, XHR performed experiments and proofread the manuscript, YLT performed experiments and collected clinical specimen, LJW performed experiments, JWJ and XWQ performed experiments and collected clinical specimen, ZZL proofread the manuscript, WR designed experiments and wrote the manuscript, LBW designed experiments and supervised the research. All authors contributed to the article and approved the submitted version.

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Tables

Table1 Correlations between ELK3 and clinicopathologic parameters in pancreatic cancer patients.

Parameters	No.	ELK3 expression		χ ²	P
	(n=70)	high (n=46)	low (n=24)		
Gender					
Male	46	30	16	0.015	0.904
Female	24	16	8		
Age (years)					
≤60	28	18	10	0.042	0.837
>60	42	28	14		
Tumor location					
Head	45	27	18	1.826	0.177
Body/tail	25	19	6		
Tumor size (cm)					
≤3	28	15	13	3.054	0.081
>3	42	31	11		
Pathologic grade					
I-II	48	28	20	3.693	0.055
III	22	18	4		
T stage					
T1-T2	61	37	24	5.388	0.020
T3	9	9	0		
N stage					
N0	42	23	19	5.590	0.018
N1	28	23	5		
M stage					
M0	62	38	24	4.712	0.03
M1	8	8	0		
AJCC stage					

0-IIA	41	23	18	4.06	0.044
IIB-IV	29	23	6		
Perineural invasion					
Yes	30	22	8	1.353	0.245
No	40	24	16		
Vascular invasion					
Yes	17	9	8	1.156	0.282
No	53	37	16		

χ^2 Test was used to test the association between categorical variables

Table2 Correlations between ZEB1 and clinicopathologic parameters in pancreatic cancer patients.

Parameters	No.	ZEB1 expression		χ^2	P
	(n=70)	high (n=41)	low (n=29)		
Gender					
Male	46	28	18	0.292	0.540
Female	24	13	11		
Age (years)					
≤60	28	16	12	0.039	0.843
>60	42	25	17		
Tumor location					
Head	45	30	15	3.403	0.065
Body/tail	25	11	14		
Tumor size (cm)					
≤3	28	15	13	0.481	0.488
>3	42	26	16		
Pathologic grade					
I-II	48	24	24	4.624	0.0315
III	22	17	5		
T stage					
T1-T2	61	35	26	0.279	0.597
T3	9	6	3		
N stage					
N0	42	19	23	7.693	0.006
N1	28	22	6		
M stage					
M0	62	34	28	3.115	0.078
M1	8	7	1		
AJCC stage					
0-IIA	41	19	22	6.100	0.014

IIB-IV	29	22	7		
Perineural invasion					
Yes	30	19	11	0.491	0.700
No	40	22	18		
Vascular invasion					
Yes	17	7	10	2.800	0.094
No	53	34	19		

χ^2 Test was used to test the association between categorical variables

Figures

Figure 1

ELK3 is elevated in pancreatic cancer tissues compared with adjacent normal tissues. (A) ELK3 expression levels from the Oncomine database. (B) ELK3 mRNA levels in GEO datasets GSE15471 (T, n = 36, N, n = 36) and GSE71989 (T, n = 14, N, n = 8). (C) ELK3 mRNA expression in the TCGA cohort (T, n = 179, N, n = 171). (D) Overall survival and relapse free survival of patients with low and high ELK3 level from the Kaplan-Meier analysis. (E) Representative IHC images of ELK3 on the TMA constructed from 70

pancreatic cancer tissues and adjacent normal tissues (scale bar: 200μm, magnification: top 50 x and bottom 200 x). (F) IHC scores of ELK3 in 70 cases of PDAC tissues with corresponding normal tissues. *P < 0.05, **P < 0.01.

Figure 2

ELK3 promotes proliferation, migration and invasion of PDAC cells in vitro. (A) Colony formation assay revealing the proliferative ability of indicated PANC-1 and MIA PaCa-2 cells. (B) EdU assay confirming the

proliferative ability of indicated PANC-1 and MIA PaCa-2 cells. (C) Representative images of wound healing assays performed with the indicated PANC-1 and MIA PaCa-2 cells. (D) Representative images of transwell migration and matrigel invasion assays performed with the indicated PANC-1 and MIA PaCa-2 cells. Biological triplicate experiments were performed for each group. All data are presented as the mean $\pm$ SD. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$.

Figure 3

ELK3 promotes tumor growth and metastasis of PDAC cells in vivo. (A) Representative images of nude mice and xenograft tumours derived from sh-ELK3/MIA PaCa-2 and sh-NC/ MIA PaCa-2 cells. (B) Tumor growth curves showing the tumor volume as monitored every three days in the control and ELK3 knockdown groups. (C) The tumors were removed and weighed after 21 days in the control and ELK3

knockdown groups. (D) Representative images of nude mice and xenograft tumors derived from Ctrl/PANC-1 and ELK3/PANC-1 cells. (E) Tumor growth curves showing the tumor volume as monitored every three days in the control and ELK3 overexpressing groups. (F) The tumors were removed and weighed after 21 days in the control and ELK3 overexpressing groups. (G) Representative photographs of pulmonary metastasis and H&E-stained lung sections in the control and ELK3 knockdown groups (scale bar: 100 μ m, magnification: 50 x). (H) Number of lung metastatic nodules was counted. (I) Representative photographs of pulmonary metastasis and H&E-stained lung sections in the control and ELK3 overexpressing groups (scale bar: 100 μ m, magnification: 50 x). (J) Number of lung metastatic nodules was counted. Biological triplicate experiments were performed for each group. All data are presented as the mean $\pm$ SD. *P < 0.05, **P < 0.01, ***P < 0.001.

Figure 4

ELK3 is crucial for TGFβ-induced EMT in PDAC. (A) Western blot analysis of E-cadherin, N-cadherin and Vimentin expression in the indicated cells. (B) Representative confocal immunofluorescence images of E-cadherin, N-cadherin and Vimentin expression in the indicated cells (scale bar: 200 μm). (C) Representative images of wound healing in sh-ELK3/PANC-1 and sh-ELK3/MIA PaCa-2 cells treated with or without TGFβ. (D) Representative images of migration and invasion in sh-ELK3/PANC-1 and sh-

ELK3/MIA PaCa-2 cells treated with or without TGFβ. Biological triplicate experiments were performed for each group. All data are presented as the mean ± SD. *P < 0.05, **P < 0.01.

Figure 5

ELK3 activates the Wnt/β-catenin signaling pathway in PDAC. (A) The effects of ELK3 on total β-catenin, nuclear β-catenin and cytosolic β-catenin levels, as detected by western blot. (B) Representative images of wound healing in ELK3/PANC-1 and ELK3/MIA PaCa-2 cells treated with or without siβ-catenin. (C)

Representative images of migration and invasion in ELK3/PANC-1 and ELK3/MIA PaCa-2 cells treated with or without siβ-catenin. Biological triplicate experiments were performed for each group. All data are presented as the mean ± SD. *P < 0.05, **P < 0.01.

Figure 6

ZEB1 activates the ELK3 transcriptional activity in pancreatic cancer. (A)The JASPAR database revealed the DNA-binding motifs of ZEB1 on the promoters of ELK3. (B) Positions and binding sequences of five

putative ZEB1 binding sites on the ELK3 promoter. (C) QRT-PCR analysis of ELK3 mRNA levels in PANC-1 and MIA PaCa-2 cells transfected with si-ZEB1 or ZEB1 overexpression plasmid. (D) Western blot analysis of ELK3 protein levels in PANC-1 and MIA PaCa-2 cells transfected with si-ZEB1 or ZEB1 overexpression plasmid, GAPDH was used as the loading control. (E, F) ChIP-qPCR experiments on five different ELK3 promoter primer using anti-ZEB1 antibody in PANC-1 and MIA PaCa-2 cells. (G) ChIP-qPCR experiments on the ELK3 promoter using anti-ZEB1 antibody in PANC-1 and MIA PaCa-2 cells transfected with si-ZEB1 or ZEB1 overexpression plasmid. (H) Schematic of wild and mutant reporter plasmids. (I) Relative luciferase activities in PANC-1 and MIA PaCa-2 cells transfected with ZEB1 overexpression plasmid. Biological triplicate experiments were performed for each group. All data are presented as the mean $\pm$ SD. *P < 0.05, **P < 0.01.

Figure 7

ELK3 is critical for the function of ZEB1 on PDAC cell proliferation and migration. (A) EdU assay analyzing the proliferative ability of PANC-1 cells. (B) Representative images of transwell migration and matrigel invasion assays performed with the indicated PANC-1 cells. (C) Representative images of wound healing assays performed with the indicated PANC-1 cells. (D) Western blot analysis of E-cadherin, N-cadherin and Vimentin expression in PANC-1 cells. (E) Western blot analysis of E-cadherin, N-cadherin

and Vimentin expression in MIA PaCa-2 cells. Biological triplicate experiments were performed for each group. All data are presented as the mean $\pm$ SD. * $P < 0.05$, ** $P < 0.01$.

Figure 8

ELK3 and ZEB1 correlate closely with pancreatic cancer clinical pathological features. (A) Representative IHC images of ZEB1 on the TMA constructed from 70 pancreatic cancer tissues and adjacent normal tissues (scale bar: 200 μ m, magnification: top 50 x and bottom 200 x). (B) IHC scores of ZEB1 in 70 cases

of PDAC tissues with corresponding normal tissues. (C) Representative IHC images of ZEB1 and ELK3 in ZEB1 high expression or ZEB1 low expression tissues (scale bar: 200 μ m, magnification: top 50 x and bottom 200 x). (D) Kaplan-Meier survival analysis of the correlation of ELK3 expression level with OS of PDAC patients. (E) Kaplan-Meier survival analysis of the correlation of ZEB1 expression level with OS of PDAC patients. (F) OS analysis based on the co-expression of ELK3 and ZEB1 in 70 PDAC patients.

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