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Original Article

FoxQ1 promoted metastatic potential of pancreatic cancer via transcriptionally activating ZEB2

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Abstract: The oncogenic role of forkhead box Q1 (foxQ1) had been clarified in multiple malignancies while its role in pancreatic cancer was not fully understood. In this study, we first confirmed the clinical significance of foxQ1 expression in pancreatic cancer. We observed that foxQ1 was overexpressed in pancreatic cancer tissues and cell lines compared with non-tumor tissues and normal pancreatic ductal cell line HPDE, respectively. By immunohistochemical assay, we found that expression of foxQ1 predicted later TNM stage and poorer survival status. Then we provided new findings that foxQ1 promoted metastatic potential of pancreatic cancer cells via transcriptionally activating zinc finger E-box binding homeobox 2 (ZEB2), a well-known transcriptional suppressor of E-cadherin. Silencing foxQ1 inhibited the migration and invasion ability of PANC-1 cells via down-regulating ZEB2 expression while overexpressing foxQ1 promoted these aggressive behaviors of ASPC1 cells via up-regulating ZEB2 level. FoxQ1 and its downstream effector ZEB2 might provide novel therapeutic strategy of pancreatic cancer.

Keywords: FoxQ1, transcriptionally activating, ZEB2, metastasis, pancreatic cancer

Introduction

Forkhead-box (FOX) proteins, which shared a conserved forkhead or winged helix domain, could bind DNA as monomers and regulate biological processes including metabolism, immunology, cell differentiation as well as neurocognitive function [1-5]. Recent literatures had revealed a close relationship between foxQ1 and cancer progression. Overexpression of foxQ1 in hepatocellular carcinoma (HCC) was correlated with larger tumor volume, higher serum α-fetoprotein level and later TNM stage [6]. FoxQ1 enhanced tumorigenicity of colorectal cancer and promoted tumor growth [7]. FoxQ1 could also regulate epithelial-mesenchymal transition (EMT) of breast cancer, HCC, non-small cell lung cancer and bladder cancer [8-12]. However, the role of foxQ1 in pancreatic cancer was still little known. Sarkar et al reported that triple-marker-positive ($CD44^+/CD133^+/EpCAM^+$) cancer stem-like cells (CSLCs) isolated from pancreatic cancer cell lines exhibited an up-regulation of foxQ1 compared with the triple-marker-negative ($CD44^-/CD133^-/EpCAM^-$) cells [13]. Silencing foxQ1 in CSLCs attenuated tumor formation and growth, suggesting the aggressive potential of foxQ1 in human pancreatic cancer, which still need further investigation.

To further illustrate the clinical significance and biological function of foxQ1 in pancreatic cancer, we evaluated the foxQ1 mediated behaviors of this deadly disease. Firstly, we investigated the expression of foxQ1 in clinical samples. It was demonstrated that foxQ1 overexpression in cancer tissues predicted advanced tumor stage and adverse outcomes of pancreatic cancer patients. Subsequently, the biological study showed that foxQ1 could regulate the metastatic potential of pancreatic cancer cells. Lastly, we demonstrated that Zinc Finger E-Box Binding Homeobox 2 (ZEB2), a transcriptional suppressor of E-cadherin, was the main downstream target of foxQ1 and responsible for foxQ1-mediated metastasis. Our new findings might facilitate the understanding of foxQ1-mediated carcinogenesis and metastasis of pancreatic cancer.

Table 1. Primer sequences used in the study

Name	Sequeces (5'-3')
Real-time PCR	
FoxQ1-F	5'-CGACTGCTTCGTCAAGGT-3'
FoxQ1-R	5'-CCGTCGGCGAAGGTGTA-3'
ZEB2-F	5'-TTCTGCGACATAAATACG-3'
ZEB2-R	5'-GAGTGAAGCCTTGAGTGC-3'
GAPDH-F	5'-GCACCGTCAAGGCTGAGAAC-3'
GAPDH-R	5'-TGGTGAAGACGCCAGTGGA-3'
Chromatin immunoprecipitation assay	
ZEB2 binding site 1-F	5'-ACAGCAGAGCATTGGTTA-3'
ZEB2 binding site 1-R	5'-TCTGTCGTAATCCAATCA-3'
ZEB2 binding site 2-F	5'-TGATTGGATTACGACAGA-3'
ZEB2 binding site 2-R	5'-ACTCCACCTTTGCTCTGA-3'
ZEB2 promoter site-directed mutagenesis	
Binding site 1 mutation-F	5'-ATATCTTTCTTacgcAGGACCTATGT-3'
Binding site 1 mutation-R	5'-ACATAGGTCCTgcgtAAGAAAGATAT-3'
Binding site 2 mutation-F	5'-TTTGAGTGATTcgcgAATTAACCATA-3'
Binding site 2 mutation-R	5'-TATGGTTAATTcgcgAATCACTCAAA-3'
siRNA sequence	
Scramble siRNA-F	5'-UUCUCCGAACGUGUCACGUTT-3'
Scramble siRNA-R	5'-ACGUGACACGUUCGGAGAATT-3'
FoxQ1 siRNA-F	5'-CCAUCAAACGUGCCUUAAA-3'
FoxQ1 siRNA-R	5'-UUUAAGGCACGUUUGAUGG-3'
ZEB2 siRNA-F	5'-GGACACAGGUUCUGAAACA-3'
ZEB2 siRNA-R	5'-CCUGUGUCCAAGACUUUGU-3'

Materials and methods

Clinical specimens

The clinical research protocol was approved by the First Affiliated Hospital of Zhengzhou University. Written informed consents were collected from all the included patients and the pathological results were confirmed by two independent pathologists. Pancreatic cancer tissues and corresponding non-tumor tissues were collected from patients who undergone pancreatic resections. Fresh specimens were cut into wedge shapes, transported with liquid nitrogen, and preserved at -80°C for Quantitative Real-time PCR (qRT-PCR) assay. Formalin soaked tissues were used for immunochemistry analysis.

Immunohistochemistry and scoring

Formalin soaked tissues were embedded by paraffin and cut at 4 μm thicknesses for HE staining. As to immunochemistry staining, sections were incubated with anti-foxQ1 (ab51340, Abcam, USA) at 4°C overnight and then incubated with horseradish peroxidase (HRP)-conjugated secondary goat anti-rabbit antibody (ab6721, Abcam, USA). Immunohistochemical staining was performed using the Dako Envision Plus System (Dako, Carpinteria, CA) according to the manufacturer's protocol.

Scoring of foxQ1 expression was performed by two independent researchers, and discrepancies were resolved by consensus with another independent researcher. The staining intensity was scored as 0 (no staining), 1 (weak staining), 2 (moderate staining), 3 (strong staining). Positive cells on each section were scored as 0 (<10%), 1 (10%-25%), 2 (26%-50%), 3 (>50%). The final score of each section was calculated by multiplying score of positive cells and staining intensity. Sections scored of 0-3 represented lower expression of foxQ1 while 4-9 represented higher expression.

Cell culture and qRT-PCR assay

Pancreatic cancer cell lines PANC-1 SW1990, BXPC3, ASPC1 and the normal immortalized human pancreatic cell line HPDE were purchased from the Type Culture Collection of the Chinese Academy of Sciences (Shanghai, China). All cells were cultured in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% fetal bovine serum (Gibco, Carlsbad, CA, USA) at 37°C under 5% CO_2 in a humidified chamber.

Trizol regent (Invitrogen) was used for total RNA extraction and synthesis of cDNA was conducted by using a two-step reverse transcription kit (TOYOBO, Japan). qRT-PCR analysis was performed with SYBR Green reagent (Applied Biosystems) according to the manufacturer's instructions. The primers used in the present study were listed in **Table 1**. The relative expression levels of target genes were examined by the following formula: $2^{-\Delta\Delta Ct}$ ($\Delta Ct = \Delta Ct^{target}$-$\Delta Ct^{GAPDH}$; $\Delta\Delta Ct = \Delta Ct^{expressing\ vector}$-$\Delta Ct^{control\ vector}$).

siRNAs, plasmids and transient transfection

siRNAs targeting foxQ1 or ZEB2 and the scramble siRNA were synthesized by GenePharma

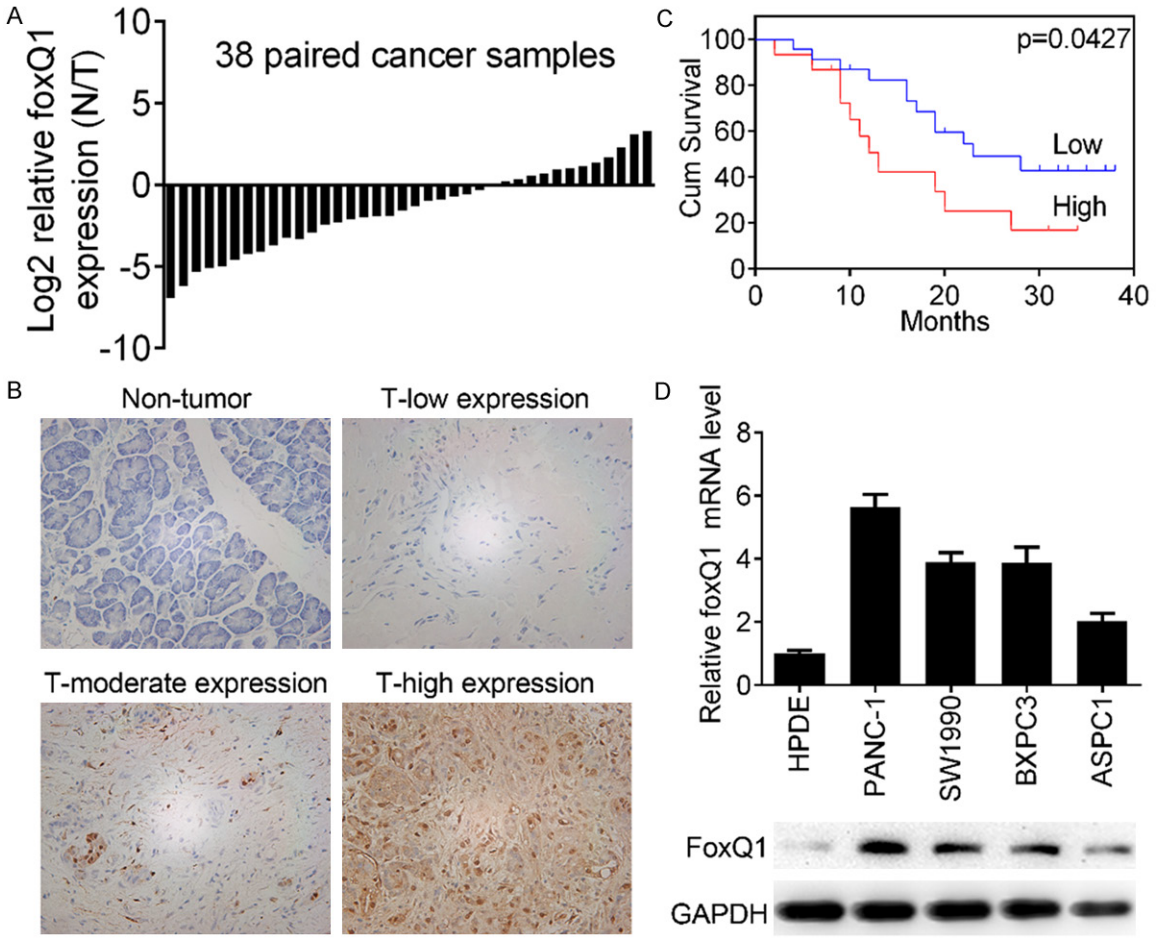

Figure 1. Clinical significance of foxQ1 expression in pancreatic cancer. A. FoxQ1 was overexpressed in pancreatic cancer tissues compared with non-tumor tissues by qRT-PCR assay. B. Expression of foxQ1 by immunohistochemistry in clinical specimens. C. Overall survival analysis between patients with low and high foxQ1 expression. D. FoxQ1 was overexpressed in PANC-1 SW1990, BXPC3 and ASPC1 cells compared with the normal immortalized human pancreatic cell line HPDE. T, tumor tissue.

(Shanghai, China). FoxQ1 and ZEB2 expressing plasmids (pcDNA3.1-foxQ1 and pcDNA3.1-ZEB2) and the empty vector were synthesized by Cyagen Biosciences, China. Cells were seeded into 6-well plate at an appropriate density and transient transfection was performed at 60% confluence by using lipofectamine 2000 (Invitrogen). RNA isolation, protein extraction and cell functional assay were conducted 48 h after transfection.

Cell migration and invasion assay

Transwell (8-μm pore size, Corning, USA) was used for evaluating cell migration and invasion ability. For migration analysis, 5×10^4 cells were plated into the non-coated top chambers. For invasion assay, the top chambers were coated with 200 mg/ml of Matrigel (BD biosciences, USA); dried overnight and 1×10^5 cells were plated in the top chamber. After incubated for 24 h, the chambers were stained with 1% crystal violet for 30 min and the migrated/invaded cells were counted with 5 random fields.

Chip-PCR assay

Chip assay was performed by using the EZ CHIP KIT (Merck Millipore). In brief, cells were cross-linked with 1% formaldehyde at 37°C for 10 min, added with Glycine Solution and washed by cold PBS. After cells were resuspended in SDS lysis buffer added with protease inhibitor cocktail and incubated for 10 min on ice, total DNA was shared to 200-1000 bp by sonication. Then the samples were incubated with anti-

Table 2. Relationship between foxQ1 expression level and clinicopathologic parameters in 38 clinical samples

Parameters	n	FoxQ1 expression (%)		*P*
		Low	High	
Age (years)		15	23	
<60	21	9	12	0.635
≥60	17	6	11	
Gender				
Male	22	8	14	0.646
Female	16	7	9	
Perineural invasion				
Yes	23	8	16	0.311
No	14	7	7	
TNM stage				
IA-IB	14	9	5	0.017
IIA-IIB	24	6	18	

foxQ1 or control IgG overnight and protein G agarose for 2 h. The immunoprecipitated DNA was retrieved from the agarose with elusion buffer and purified for PCR assay. The primers were listed in **Table 1**.

Luciferase activity assay

Dual Luciferase Assay Kit (Promega, USA) was used for evaluating the transcriptional activity of ZEB2 according to the manufacturer's instructions. Briefly, transfected cells were lysed, centrifuged and the supernatants were collected for analyzing according to the protocol. RLU value of firefly luciferase assay was normalized according to the Renilla activity.

Western blot assay

Proteins were extracted by RIPA buffer (Beyotime Biotechnology, China) containing protease inhibitor and phosphatase inhibitor (Selleckchem, China). Proteins were separated with SDS-PAGE and transfected onto a PVDF membrane (Merck Millipore). Blots were incubated with anti-foxQ1, anti-ZEB2 (ab138222, Abcam, USA), anti-E-cadherin (#3195, Cell Signaling Technology) and anti-GAPDH (ab8245, Abcam, USA) overnight, respectively. Then the purpose bands were incubated with corresponding HRP-conjugated secondary goat anti-rabbit antibody (ab6721, Abcam, USA) or goat anti-mouse antibody (ab6789, Abcam, USA). Proteins were visualized by Dura SuperSignal Substrate (Pierce, USA).

Statistical analysis

Statistical analyses were conducted by using SPSS 16.0 software. Student's t-test was performed to analyze the data of two groups including cell migration, invasion, luciferase activity, ZEB2 mRNA level and relative ZEB2 promoter enrichment. Pearson χ^2 test was applied to analyze the relationship between foxQ1 expression and clinicopathologic parameters. Kaplan-Meier survival curves were compared by log-rank test. All experimental results were repeated at least three times and were shown as mean ± standard deviation (s.d.).

Results

Clinical significance of foxQ1 expression in pancreatic cancer

As the result of qRT-PCR assay, 26 out of 38 cases showed up-regulation of foxQ1 in cancer tissues while only 12 cases showed down-regulation (**Figure 1A**). Then we evaluated the relationship between foxQ1 level and the clinicopathological features by scoring the result of immunochemistry assay (**Figure 1B**), which showed a positive relationship between foxQ1 expression and TNM stage (**Table 2**). Furthermore, high expression of foxQ1 indicated poorer survival of pancreatic cancer patients compared with low expression patients (**Figure 1C**). We also proved that foxQ1 was highly expressed in a couple of pancreatic cancer cell lines compared with the normal immortalized human pancreatic cell line HPDE by qRT-PCR and western blot assay (**Figure 1D**).

FoxQ1 promoted migration and invasion of pancreatic cancer cells

Given the clinical significance of foxQ1 in pancreatic cancer, we then asked whether foxQ1 could regulate the biological role of pancreatic cancer cells. Silencing foxQ1 in PANC-1 cells, which showed higher foxQ1 level, reduced the migrated and invaded cells through transwell (**Figure 2A** and **2B**). Consistently, overexpression of foxQ1 in ASPC1 cells, which had a lower expression of foxQ1, promoted the migration and invasion ability (**Figure 2C** and **2D**). Additionally, the protein level of ZEB2, a transcrip-

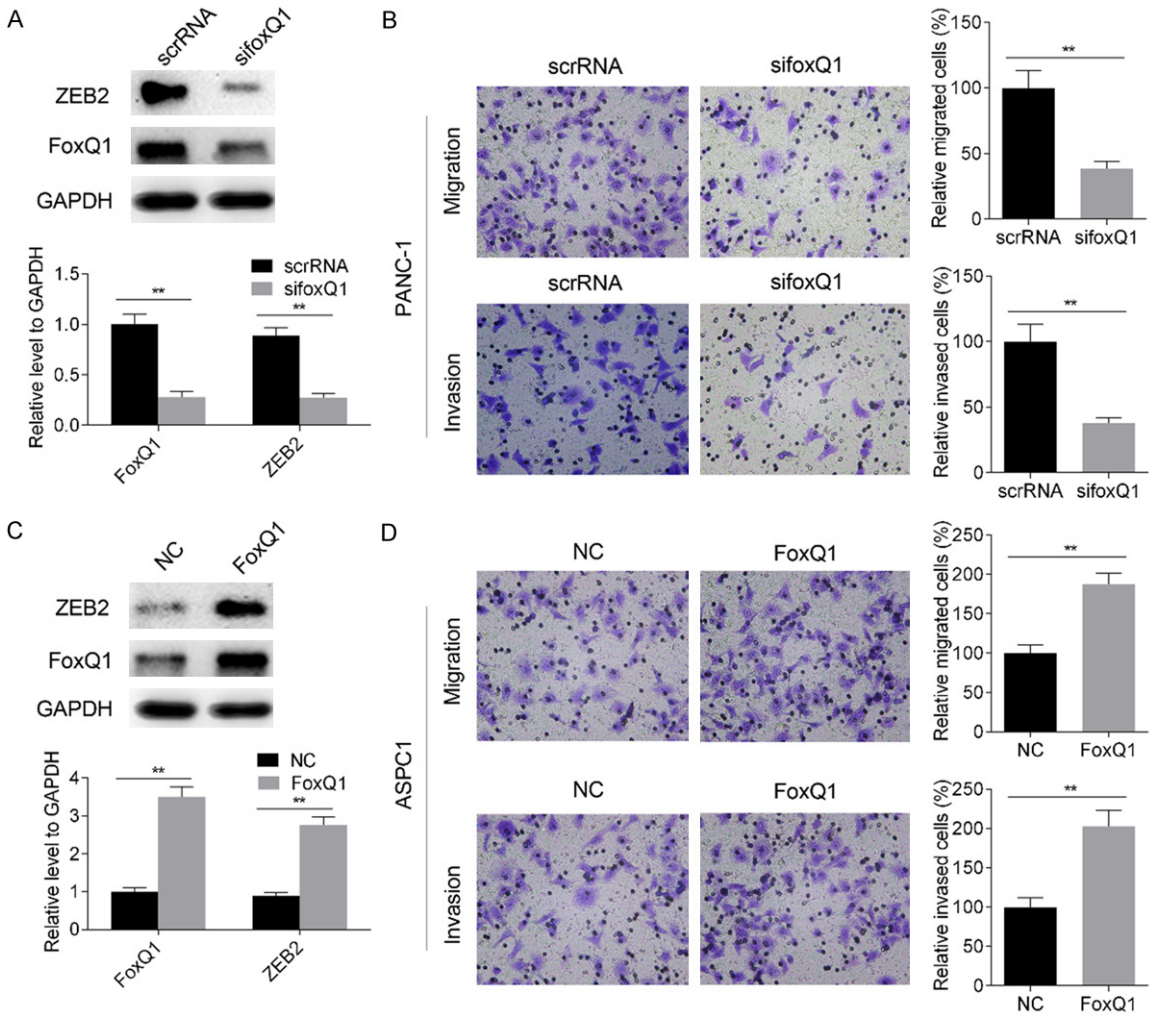

Figure 2. FoxQ1 regulated migration and invasion of pancreatic cancer cells. A. Depletion of foxQ1 reduced the protein level of ZEB2. B. Depletion of foxQ1 inhibited the migration and invasion ability of PANC-1 cells. C. Overexpression of foxQ1 promoted the protein level of ZEB2. D. Overexpression of foxQ1 promoted the migration and invasion ability of ZEB2. **P*<0.05, ***P*<0.01.

tional repressor of E-cadherin, was reduced after silencing foxQ1 while foxQ1 overexpression promoted the level of ZEB2 (**Figure 2A** and **2C**).

FoxQ1 could transcriptionally activate ZEB2 expression

To further clarify the mechanisms behind the aggressive behaviors, we focused on how foxQ1 regulated the protein level of ZEB2. The result of qRT-PCR assay indicated that silencing foxQ1 reduced the mRNA level of ZEB2 while foxQ1 overexpression promoted the mRNA level of ZEB2 (**Figure 3A**). Two effective foxQ1 binding sites at ZEB2 promoter region were confirmed by a recent study and we verified the binding ability of both sites by chip assay [10]. As indicated in **Figure 3B** and **3C**, foxQ1 could specifically bind the both site of ZEB2 in PANC-1 cells. It is noteworthy that the result of luciferase analysis revealed that mutation of binding site 2 showed more significant down-regulation of transcriptional ability compared with mutation of binding site 1, indicating that binding site 2 was the core activation region (**Figure 3D**). These results suggested that foxQ1 could transcriptionally activate ZEB2 expression.

ZEB2 was responsible for foxQ1 induced aggressive behaviors

Given that foxQ1 could activate ZEB2 expression and regulate the metastatic ability of pancreatic cancer cells, we then asked whether

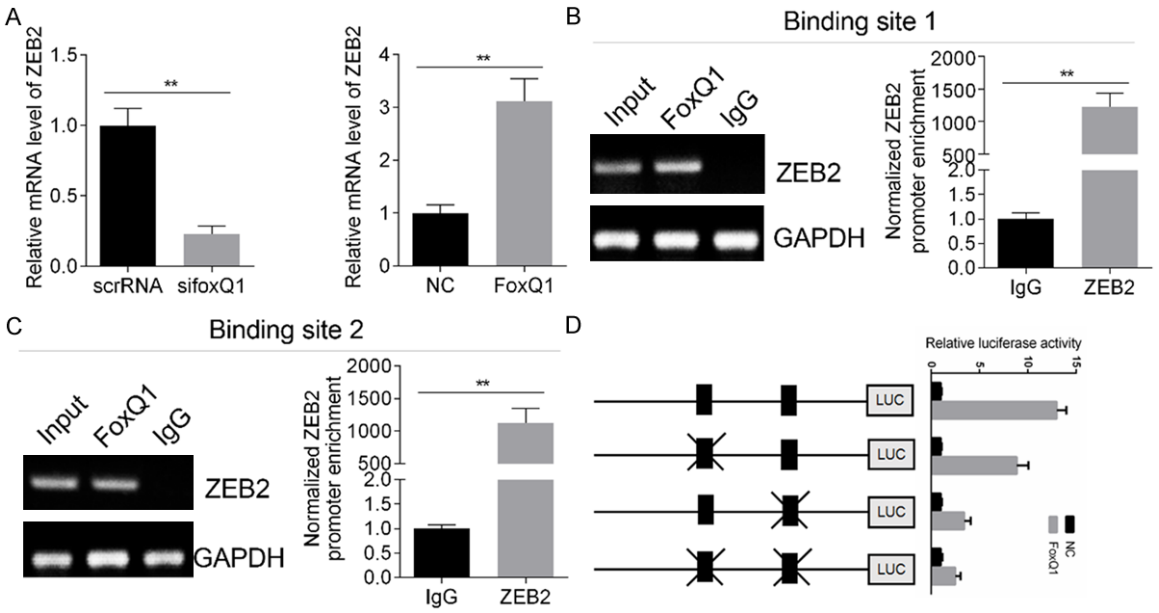

Figure 3. FoxQ1 could transcriptionally activate ZEB2 expression. A. Silencing foxQ1 reduced the mRNA level of ZEB2 while foxQ1 overexpression promoted the mRNA level of ZEB2. B, C. ChIP assay demonstrated the direct binding of foxQ1 to the both sites of ZEB2 promoter. D. Selective mutation analysis identified only one foxQ1-responsive region in the ZEB2 promoter. **P*<0.05, ***P*<0.01.

ZEB2 was responsible for foxQ1 induced aggressive behaviors. As shown in **Figure 4A** and **4B**, Ectopic expression of ZEB2 expressing plasmids, but not control plasmids, significantly rescued the migration and invasion of foxQ1-depleted PANC-1 cells. Furthermore, Expression of foxQ1 in ASPC1 cells significantly promoted cell migration and invasion that was inhibited by the depletion of ZEB2. (**Figure 4C** and **4D**) These results suggested that ZEB2 was a downstream co-effector of foxQ1 in pancreatic cancer.

Discussion

Pancreatic cancer remains one of the most lethal malignancies with a constant 5-year survival less than 10% [14]. Smoking, aging and some genetic or epigenetic disorders are potential risk factors of pancreatic cancer while the primary causes are still elusive [15-17]. Current treatment strategies for pancreatic cancer included surgical treatment and chemotherapy. 15%-20% pancreatic cancer patients could get surgical resection and had a relatively higher 5-year survival around 20%, while the majority of patients were unresectable and could only get palliative treatment including chemotherapy or radiotherapy [18-21].

Recent studies had revealed several acquired mutations of pancreatic cancer including K-*ras*, *HER2* and *AKT2* [22, 23]. Mutation status of K-*ras* had even been proposed as an early detection index. In addition, the pathogenesis of pancreatic cancer had also involved in disorders of multiple tumor-suppressor and genome-maintenance genes. Tumor suppressor *p16* is inactivated in more than 90% pancreatic cases and loss of *p53* function was a late event during the carcinogenesis of multiple malignancies [24, 25]. Targeted therapy based on several potential “drugable” targets had entered the clinical trial stage and showed improvement of survival status [26, 27]. Thus, further clarification of the molecular mechanisms of pancreatic cancer, especially the metastatic process, might accelerate the development of targeted drugs.

The clinical significance of foxQ1 in pancreatic cancer had rarely been discussed before. In the current work, we observed an up-regulation of fxoQ1 in pancreatic cancer samples compared with non-tumor tissues and overexpression of fxoQ1 was correlated with advanced tumor stage and poorer outcomes, suggesting that fxoQ1 might be an oncogene in pancreatic cancer. Tumor metastasis is the most common

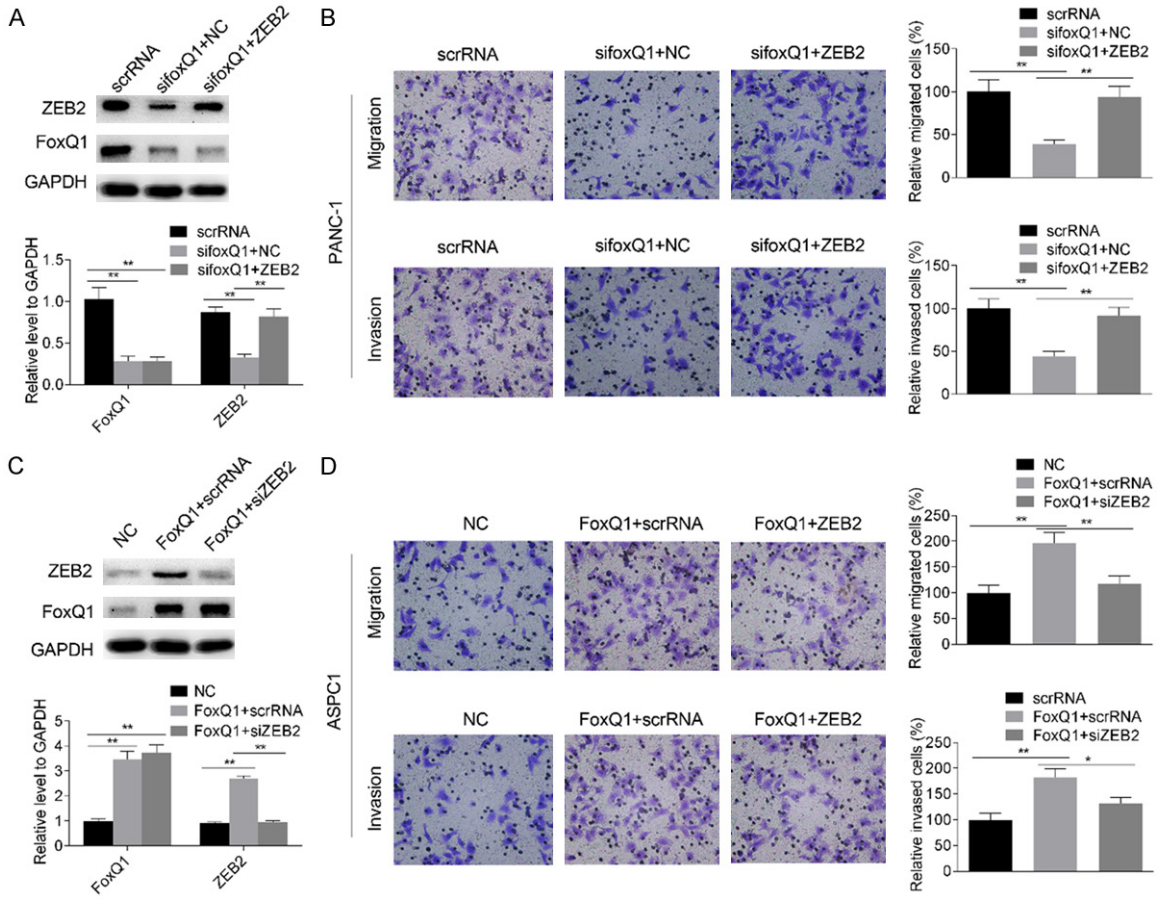

Figure 4. FoxQ1 regulated cell migration and invasion through ZEB2. A, B. Ectopic expression of ZEB2 expressing plasmids, but not control plasmids, significantly rescued the migration and invasion of foxQ1-depleted PANC-1 cells. C, D. Expression of foxQ1 in ASPC1 cells significantly promoted cell migration and invasion that was inhibited by the depletion of ZEB2. **P*<0.05, ***P*<0.01.

death-related causes of pancreatic cancer and then we explored the potential roles of fxoQ1 on cell migration and invasion. The biological studies revealed that foxQ1 promoted cell migration and invasion as well as the protein level of ZEB2, a well-known metastatic-related protein. However, how fxoQ1 regulated ZEB2 level and whether foxQ1 induced malignant behaviors through ZEB2 still need further study.

Epithelial-mesenchymal transition (EMT), a dynamic process through which epithelia cells obtained the mesenchymal phenotypes and thus exhibited strong migratory and invasive abilities, was characterized by the up-regulation of mesenchymal markers including slug, snail, vimentin as well as ZEB1/ZEB2 and the down-regulation of epithelia markers including E-cadherin. Aberrant expression of E-cadherin might induced by the alterations of several transcriptional factors and ZEB2 was a well-recognized transcriptional inhibitor of E-cadherin [28, 29]. In the present work, we observed that foxQ1 promoted cell migration and invasion through transcriptionally activating ZEB2. Re-expression of ZEB2 in sifoxQ1 cells rescued the migration and invasion ability and silencing ZEB2 in foxQ1 overexpression cells could partly inhibit the migration and invasion ability. These evidences provided a preliminary explanation of foxQ1 induced aggressive behaviors, which still need more investigations.

In conclusion, we found that foxQ1 played a tumor-inducing role in pancreatic cancer. Although foxQ1's function in cancer has not been fully understood, we provided novel mechanisms of foxQ1 induced pancreatic cancer metastasis. FoxQ1 had the potential value

to be developed as a therapeutic target of pancreatic cancer.

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Disclosure of conflict of interest

None.

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