Original Article
SRPX2 promotes cell migration and invasion via FAK dependent pathway in pancreatic cancer

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Abstract: Sushi repeat-containing protein, X-linked 2, abbreviated as SRPX2, is a candidate downstream target protein for E2A-HLF and involved in disorders of language cortex and cognition. Recent studies have demonstrated that elevated SRPX2 exhibits crucial roles in gastric cancer, however, underlying clinical significance and biological function of SRPX2 in pancreatic ductal adenocarcinoma (PDAC), remains unclear. Data from Oncomine database showed that higher SRPX2 expression is more commonly observed in PDAC compared with normal pancreatic duct, similar results were also found in 12 matched PDAC tissue samples, 7 PDAC cell lines and a tissue microarray containing 81 PDAC specimens as demonstrated by real-time quantitative PCR and immunohistochemistry, respectively. Besides, higher SRPX2 expression was closely correlated with advanced TNM stage. Silencing of endogenous SRPX2 expression reduced abilities of cell migration and invasion of PDAC cells. Further studies revealed that SRPX2 expression in PDAC tissues significantly correlated with the phosphorylation levels of FAK, indicating that FAK dependent pathway may be account for the effect of SRPX2 on cell migration and invasion in PDAC. Collectively, this study reveals that frequently elevated SRPX2 contributes to cell migration and invasion in PDAC and SRPX2-related pathways might be a potential therapeutic target for PDAC.

Keywords: SRPX2, pancreatic ductal adenocarcinoma, migration, invasion

Introduction

Pancreatic cancer remains the most malignancies with a 5-year survival rate remained at 3% to 5% for the past three decades [1]. Pancreatic ductal adenocarcinoma (PDAC) is the most common type of pancreatic cancer with 95% of reported cases [2]. Unfortunately, due to the rapid local and systemic spread of PDAC cells, most cases are ineligible to surgical resection by the time of diagnosis [3]. Besides, all currently available treatments, including surgical resection and chemotherapy, fail to improve patients' prognosis. Therefore, it's urgent to develop novel therapeutic approaches or find new prognostic factors to improve the poor prognosis of PDAC patients.

Sushi repeat-containing protein, X-linked 2 (SRPX2), is a secreted protein that modulates synapse density in dissociated hippocampal neurons [4]. FoxP2, an activity-independent transcript-ion factor, regulates synaptogenesis through SRPX2 [5]. Mutation of the SRPX2 gene is responsible for human patients suffering from rolandic seizures with associated oral and speech dyspraxia and mental retardation [6, 7]. SRPX2 contains three sushi domains, also known as complement control protein (CCP) domain, had been discovered as regulators of neuronal development and the immune system in vertebrates [8]. For its molecular and biological functions, SRPX2 was found to directly interact with urokinase plasminogen activator receptor (uPAR) and pathogenic mutation of SRPX2 enhanced the SRPX2/uPAR ligand–receptor interaction [9]. Recently, it has been demonstrated that SRPX2, as a novel chondroitin sulfate proteoglycan, is overexpressed in gastric cancer and promotes cellular migration and adhesion [10]. Besides, SRPX2 is also frequently upregulated in colorectal cancer; methylation of particular CpGs is closely linked to SRPX2 gene expression and miR-149 contributes to the regulation of the

SRPX2 transcript level [11]. However, possible roles of SRPX2 in pancreatic cancer remain unclear.

In the present study, we firstly observe that elevated expression of SRPX2 in both PDAC cell lines and clinical specimens. Suppressed SRPX2 expression results in decreased cell migration and invasion abilities. Moreover, our results indicate that SRPX2 expression is closely correlated with phosphorylation levels of FAK, thus these two molecules dependent pathways plays vital roles in PDAC progression.

Materials and methods

Cell culture

Human PDAC cell lines AsPC1, Capan2, CFPAC1, HPAC, PANC1, SW1990 and the non-malignant hTERT-HPNE were all obtained from American Type Culture Collection. Cells were cultured in specific media supplemented with 10% (v/v) fetal bovine serum (FBS) and 1% antibiotics at 37°C in a humidified incubator under 5% CO_2 condition.

Clinical tissue samples and immunohistochemistry

A total of 12 paired freshly frozen PDAC tissues and corresponding noncancerous tissues were collected from Department of oncology, The First Affiliated Hospital of BengBu Medical College, China. The tissue microarrays contained 81 cases of PDAC tissues and 44 cases of normal pancreas tissues were purchased from Shanghai Outdo Biotech Inc. Tissue sections were deparaffinized and rehydrated. After neutralization of endogenous peroxidase and preincubated in blocking serum, slides were then incubated overnight at 4°C with primary antibody (SRPX2, Proteintech; phospho-FAK, Abcam). After washing in phosphate-buffered saline (PBS) for three times, the sections were labeled by HRP (rabbit) second antibody for 1 hour and again washed three times with PBS. The reaction products were visualized with 3,3'-diaminobenzidine tetrahydrochloride and counterstained by hematoxylin. Scoring was conducted according to the ratio of positive cells: 0-5% scored 0; 6%-35% scored 1; 36%-70% scored 2; more than 70% scored 3 and staining intensity with 0 as negative (0), weak (1), moderate (2) and strong (3). The final score was designated as low or high expression group using the percent of positive cell score × staining intensity score as follows:

low expression was defined as a total score < 4 and high expression with a total score ≥ 4. The scoring by the pathologists was done in a blinded manner.

siRNA transfection

siRNAs against SRPX2 were designed and synthesized from GenePharma (Shanghai, China). For transfection, PANC1 or SW1990 cells were seeded into a 6-well plate at a density of 2 × 10^5 cells/well. When the cells were 70% confluent, 20 nmol/L siRNAs targets against SRPX2 or a scrambled RNA negative control were tranfected into cells using Lipofectamine-2000 (Invitrogen) for 48 hours. The interference efficiency was detected by western blotting.

Quantitative real-time PCR

RNA was harvested from PDAC cell line or frozen tissue samples using Trizol reagent (Takara, Japan) and reverse transcription were peformed using the PrimeScript RT-PCR kit (Takara, Japan). Quantitative real-time PCR was performed with SYBR Green Master Mix (Takara, Japan). Specific primer sequences used were as follows: SRPX2: forward: 5'-TAGTGGCACTTACACCTGCAC-3', reverse: 5'-ATTCGGCTGCGATCACCTTC-3'; β-actin: forward: 5'-CATGTACGTTGCTATCCAGGC-3', reverse: 5'CTCCTTAATGTCACGCACGAT-3'.

Western blotting

Total cell lysates were prepared using RIPA buffer containing protease inhibitor (Beyotime, China). Whole cell extracts were separated on SDS-polyacrylamide gel and then transferred to nitrocellulose membrane (Bio-Rad, USA). The membranes were then consecutively incubated with specific primary antibodies (SRPX2 and β-actin, Proteintech) and species-specific secondary antibodies after blocked with 5% (v/v) nonfat-dried milk. The immunoreactive proteins were visualized by Odyssey imaging system (LI-COR Biosciences, Lincoln, NE).

In vitro cell migration and matrigel invasion assays

For cell migration assay, 1 × 10^4 cells were seeded into the upper compartment of the transwell inserts (Millipore, USA) and the bottom chamber was placed in medium containing 5% FBS (v/v). Cells remaining in the top cham-
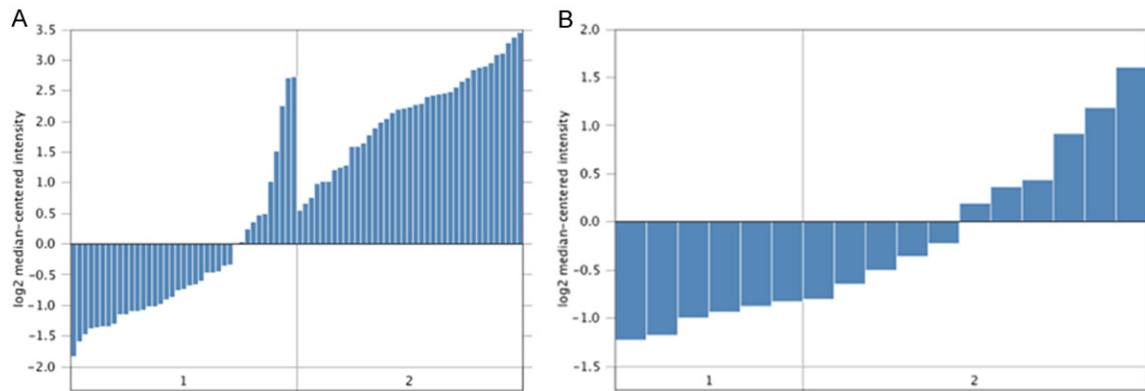
Figure 1. SRPX2 expression is increased in PDAC at mRNA level. SRPX2 expression in Badea pancreas (A) and Segara pancreas (B) grouped by normal pancreatic duct (1) and PDAC (2) derived from Oncomine database.
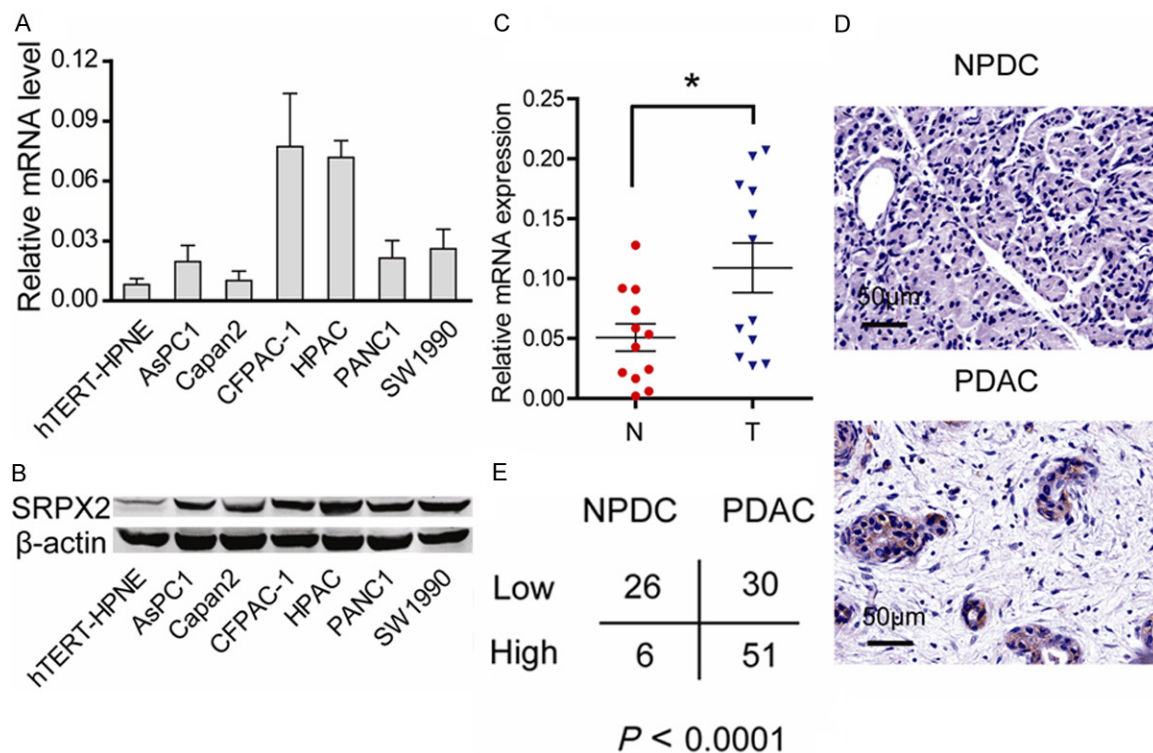
Figure 2. Expression pattern of SRPX2 in PDAC cell lines and clinical specimens. SRPX2 levels were detected in six PDAC cell lines as well as the nonmalignant hTERT-HPNE cells by quantitative real-time PCR (A) and immunoblotting (B). (C) Decreased SRPX2 mRNA expression in 12 matched tumor (T) and non-tumor tissues (N) was detected by quantitative real-time PCR. (D) Representative images of the SRPX2 expression in PDAC and normal pancreatic ductal cell (NPDC). (E) SRPX2 protein was significantly up-regulated in PDAC compared with NPDC.

bers were removed and cells migrated to the lower membrane of the inserts were fixed and stained by 0.1% (w/v) crystal violet. For matrigel invasion assay, 100 μl matrigel (BD Bioscience, USA) was placed into the top chamber of transwell inserts and 2×10^4 cells in 200 μl were seeded onto the matrigel. Cells were incubated at 37°C and allowed to invade through matrigel

for 48 hours and treated according to the procedure described above.

Cell viability assay

Cells were resuspended and seeded into a 96-well plate at 2000 cells per well with 100 μl culture medium supplemented in the presence of

Table 1. Correlations between SRPX2 expression and clinico-pathologic features in patients with PDAC

Variable		Expression of SRPX2		P value
		Low (n = 30)	High (n = 51)	
Age	≤ 60 years	14 (36.84)	24 (63.16)	0.973
	> 60 years	16 (37.21)	27 (62.79)	
Gender	Female	16 (33.33)	32 (66.67)	0.405
	Male	14 (42.42)	19 (57.58)	
Tumor size	≤ 4 cm	21 (41.18)	30 (58.82)	0.314
	> 4 cm	9 (30.00)	21 (70.00)	
Tumor location	Head	21 (35.00)	39 (65.00)	0.521
	Body + tail	9 (42.86)	12 (57.14)	
TNM stage	I	22 (55.00)	18 (45.00)	**0.001**
	II-III	8 (19.51)	33 (80.49)	
Neuronal invasion	Yes	13 (31.71)	28 (68.29)	0.315
	No	17 (42.50)	23 (57.50)	

The bold number represents the P-values with significant differences.

10% FBS (v/v) and cultured overnight. The cell viability was quantified by Cell Counting Kit-8 (CCK8, Dojindo, Japan) following the manufacturer's protocols. The absorbance of each sample was measured at 450nm using a Power Wave XS microplate reader (BIO-TEK).

Apoptosis assay

Cells were seeded in 6-well plates at 3×10^5 cells per well in the absence of 10% FBS for 72 hours. Adherent cells were detached with 0.25% trypsin without EDTA in 1 × PBS and harvested in complete DMEM medium and centrifuged at 1000 rpm for 5 minutes. Each of the cells were washed with 1 × PBS and stained with propidium iodide and Annexin V-FITC (BD Pharmingen) following the manufacturer's instructions. The apoptotic cells were measured by flow cytometry.

Statistical analysis

Data were presented as the means ± standard error of the mean (SEM). Statistical analyses and graphical representations were performed with SPSS 16.0 (SPSS Inc.; Chicago, USA) and GraphPad Prism 5 (San Diego, CA) software. Correlation of SRPX2 expression with clinico-pathologic parameters was evaluated by chi-square test. The student's t-test was used for comparison between groups. A Spearman rank correlation coefficient test was carried out for testing the association between ordinal vari-

ables. Values of $P < 0.05$ were considered statistically significant.

Results

Elevated expression of SRPX2 in PDAC

To observe the expression pattern of SRPX2 in PDAC, we first searched the SRPX2 expression level in two independent microarray datasets of PDAC from Oncomine database [12, 13]. As shown in **Figure 1**, the mRNA expression levels of SRPX2 were frequently elevated in tumor tissues compared with normal pancreatic duct. In this study, the expression of SRPX2 at mRNA level was up-regulated in all of six PDAC cell lines compared with the non-malignant hTERT-HPNE cells (**Figure 2A**). This trend was also confirmed by protein expression as shown by western blotting (**Figure 2B**). In addition, 12 paired PDAC and matched noncancerous pancreas tissues were collected to detect expression of SRPX2 at the mRNA level using real-time quantitative PCR. Consistent with the results from Oncomine database, SRPX2 expression was significantly up-regulated in PDAC tumor tissues compared with corresponding non-tumor tissues (**Figure 2C**). A tissue microarray (TMA) containing 81 cases of PDAC samples and 44 cases of normal control samples was analyzed using immunohistochemical staining; compared with normal pancreatic ductal cell (NPDC), SRPX2 protein was significantly up-regulated in PDAC tumor tissues (**Figure 2D**, **2E**). Besides, high expression of SRPX2 was remarkably associated with advanced TNM stage (P = 0.001), no significant diffeence was found in age, gender, tumor size, tumor location and neuronal invasion using the Chi-square test (**Table 1**). Taken together, these results above suggest that upregulated expresion of SRPX2 may promote tumor progression in PDAC.

Silencing of SRPX2 inhibits cell migration and invasion in vitro

Given SRPX2 expression was closely correlated with TNM stage but not tumor size, we hypothesized that SRPX2 contributes to tumor invasion and metastasis. To test this hypothesis,
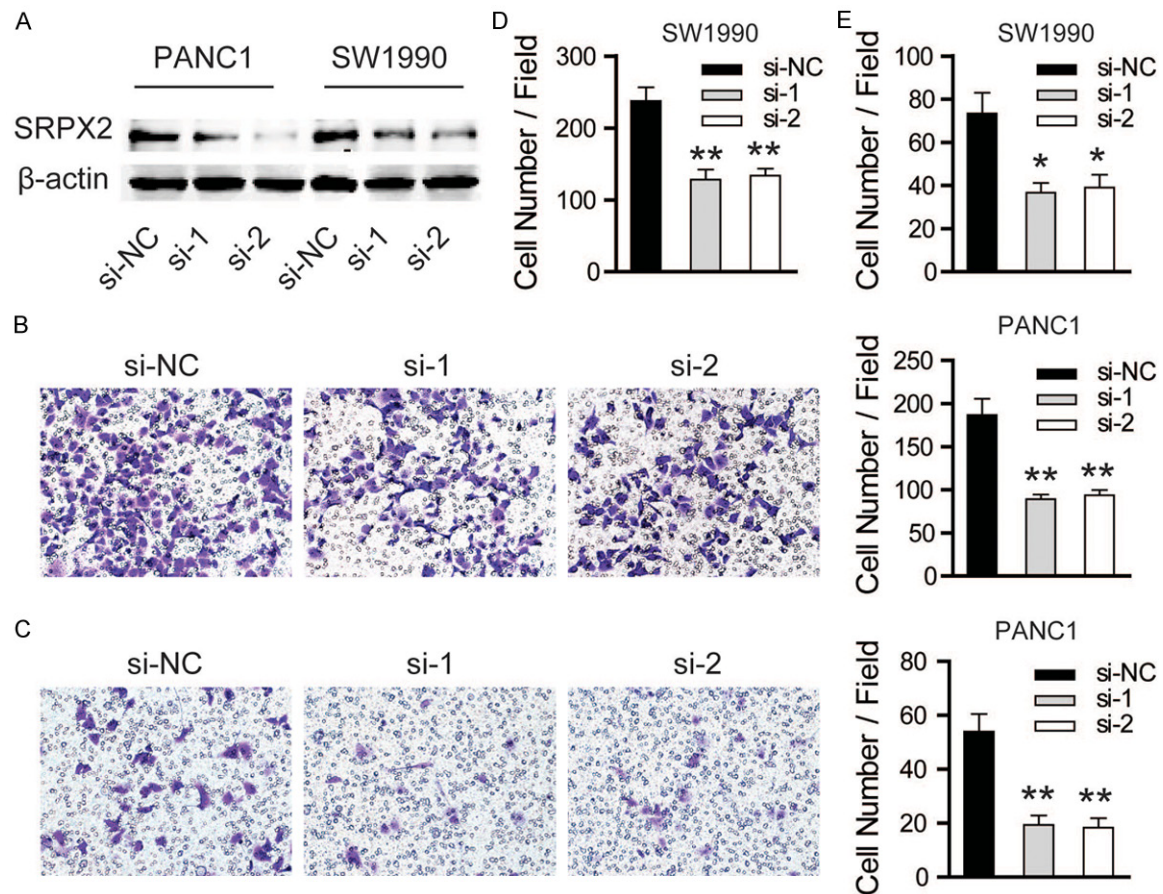
Figure 3. Knockdown of SRPX2 inhibits cell migration and invasion in PANC1 and SW1990 cells. (A) The expression level of SRPX2 was detected by western-bolting in PANC1 and SW1990 cells after siRNA treatment. (B) Effect of SRPX2 knockdown on cell migration of PANC1 cells (sh-NC versus si-1 or si-2, **, $P < 0.01$). (C) Effect of SRPX2 knockdown on cell invasion of PANC1 cells (sh-NC versus si-1 or si-2, **, $P < 0.01$). Cell migration (D) and invasion abilities (E) of SW1990 cells after SRPX2 was knockdown (si-NC versus si-1 or si-2, *, $P < 0.05$; **, $P < 0.01$). Data are representative of three independent experiments.

two PDAC cell lines, PANC1 and SW1990, were transfected with two siRNAs (si-1 and si-2) target SRPX2; the protein expression of SRPX2 was markedly decreased after both siRNAs treatment (**Figure 3A**). Transwell model was performed to analyze the ability of cell migration and invasion. Migration assay showed that the number of migratory cells in siRNA treated group was dramatically decreased compared with those in the negative control group in PANC1 cells (**Figure 3B**). In the invasion assay, the invaded cells were also decreased significantly after SRPX2 knockdown in PANC1 cells (**Figure 3C**). Meanwhile, similar results were also found in SW1990 cells (**Figure 3D**, **3E**). Furthermore,cell proliferation and cell apoptosis assay were peformed after SRPX2 knockdown; consistent with former clinical data, no significant difference was found in the cell viability or cell apoptotic

ratio between siRNA treated group and negative control group (**Figure 4**). Collectively, above data indicate that SRPX2 prominently favors cell migration and invasion in PDAC.

Elevated SRPX2 correlates increased phosphorylation levels of FAK

Focal adhesion kinase (FAK) is a non-receptor protein tyrosine kinase that plays crucial roles in signalling by growth factors and extracellular matrix. Indeed, FAK plays a central role in cell spreading and migration [14]. Previous study in gastric cancer has demonstrated that FAK activity was stimulated by SRPX2 protein treatment. To determine whether SRPX2 correlates the phosphorylation of FAK in PDAC patients, we performed immunohistochemical staining to determine SRPX2 and phosphor-FAK levels
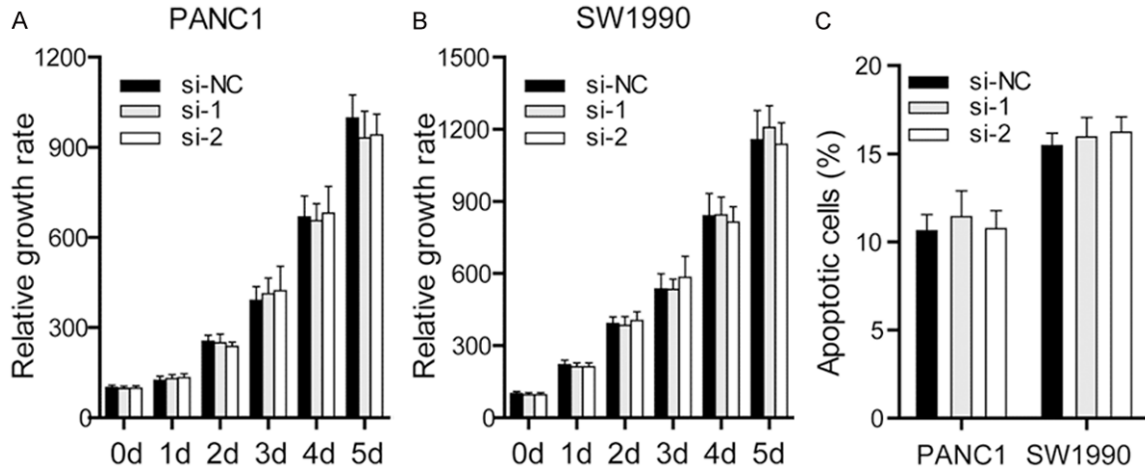
Figure 4. Effect of SRPX2 on cell proliferation and cell apoptosis. Sliencing of SRPX2 exhibits minimal effect on cell viability of PANC1 (A) and SW1990 (B) cells. (C) Effect of SRPX2 knockdown on cell apoptosis induced by serum deprivation of PANC1 and SW1990 cells.

		SRPX2				
		-	+	++	+++	Total
	-	6	1	2	2	11
	+	7	4	8	4	23
p-FAK	++	4	4	12	11	31
	+++	3	1	5	7	16
	Total	20	10	27	24	81

Spearman rank correlation test

rs = 0.324 P = 0.003

Figure 5. Elevated SRPX2 correlates increased phosphorylation levels of FAK. Statistical analysis of immunohistochemical results of SRPX2 and p-FAK expression in 81 human PDAC surgical samples. P values were calculated by the Spearman rank correlation test.

in a cohort of 81 PDAC specimens. As shown in **Figure 5**, SRPX2 expression levels in PDAC tissues significantly correlated with the levels of phosphor-FAK (r = 0.324, P = 0.003). Taken together, these data indicate that FAK signaling pathway is involved in cell migration and invasion induced by SRPX2 in PDAC.

Discussion

The aim of this study is to investigate the expression pattern of SRPX2 and its possible biological functions in PDAC. Several previous studies have demonstrated that SRPX2 expression is upregulated in various types of tumor tissues compared with adjacent normal tissues, including gastric cancer and colorectal cancer [10, 11]. Consistent with their results, our findings reveal that the expression of SRPX2 is elevated at both mRNA and protein level in PDAC Subsequent studies show that SRPX2 contributes to PDAC cell migration and invasion through FAK dependent pathway.

A previous study has demonstrated that SRPX2 is a novel secreted chondroitin sulfate proteoglycan (CSPG) [10]. Given the crucial functions of CSPGs in tumor progression and overexpressed SRPX2 in PDAC, it's reasonable to speculate that SRPX2 has an implication on tumor development in PDAC. To determine the underlying functions of SRPX2, assays of cell proliferation, apoptosis, migration and invasion were performed after SRPX2 was interfered. Similar to the results in gastric cancer that SRPX2-introduced cells or SRPX2-conditioned medium enhances cell migration but not cell growth [15], our data indicate that cell migration and invasion were significantly inhibited after silencing of SRPX2 and no significant difference was found in cell proliferation or apoptosis in PDAC cell lines. It is possible that high SRPX2 expression in PDAC or other types of cancer promotes cell migration and invasion through a specific cell signaling pathway.

SRPX2 has been previously shown to be a mediator of angiogenesis through its interac-

tion with uPAR [9] and a key molecule involved in cell migration in gastric cancer through a FAK dependent pathway [15]. It has also been reported that both the sushi domains and hyaline domain of SRPX2 have a function involved in cell mobility and migration. Moreover, the effect of SRPX2 on cell migration and invasion would also be explained by its molecular evolution: closest to the selectin family, which functions as an enhancer of tumor metastasis [16]. Here we assess the relationship between SRPX2 expression and the phosphorylation level of FAK and find that SRPX2 increased the phosphorylation level of FAK, suggesting that SRPX2 overexpression promoted cell migration and invasion through FAK signaling. Absolutely, the clear molecular mechanism of the function of SRPX2 in PDAC remains to be further demonstrated. Besides, SRPX2 is a secreted protein and may be detected in patients' serum; consideration of lacking effective early prognostic indicator in PDAC, further studies should be performed to determine the prognostic value of SRPX2 in PDAC.

In summary, this study shows that SRPX2 expression is remarkably upregulated in PDAC compared with adjacent normal tissues. Further data suggest that elevated level of SRPX2 promotes cell migration and invasion, which at least partially account for the increased phosphorylation of FAK. Although the detailed mechanism remains to be clarified, our study provides a further consideration the crucial biological functions of SRPX2 in PDAC.

Disclosure of conflict of interest

None.

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