Pathology - Research and Practice 215 (2019) 152564

Contents lists available at ScienceDirect

Pathology - Research and Practice

journal homepage: www.elsevier.com/locate/prp

Original article

Stress-induced phosphoprotein 1 promotes pancreatic cancer progression through activation of the FAK/AKT/MMP signaling axis

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ARTICLE INFO

Keywords:
STIP1
Stress-induced phosphoprotein 1
Pancreatic cancer
PANC
Metastasis
Migration
Invasion
MMP2
MMP9

ABSTRACT

Background: Dependent on the extent of adenosine triphosphate (ATP) hydrolysis and/or ATP/ADP exchange, the stress-induced phosphoprotein 1 (STIP1) mediates molecular interaction and complex formation between the molecular chaperones heat shock protein (Hsp)70 and Hsp90. The overexpression of STIP1 is increasingly being documented in various human malignancies, including ovarian, cholangiocellular, renal and gastric cancers. However, the role of STIP1 in pancreatic cancer (PANC) and probable molecular mechanism remains largely unexplored.

Methods & results: In the present study, using clinical samples (n = 88) and human PANC cell lines PANC-1, Capan-2, SW1990, and BxPC-3, we demonstrated that STIP1 is aberrantly expressed in human PANC tissues or cell lines compared to adjacent non-tumor pancreas samples or human pancreatic duct epithelial cells (HPDEC), respectively. Clinicopathological correlation studies revealed significant positive correlation between high STIP1 expression and lymph node involvement ($p = 0.001$), cancer metastasis ($p = 0.002$), microvascular invasion ($p = 0.002$), advance TNM stage ($p = 0.024$), perineural invasion (PNI; $p = 0.013$), and cancer-related death ($p = 0.002$) among patients with PANC. Univariate and multivariate analyses indicate that STIP1overexpression is an independent prognostic factor of PANC. Furthermore, STIP1 knockdown significantly inhibit the migration and invasive ability of PANC-1 and SW1990 cells, while downregulating N-cadherin and Vimentin, but upregulating E-cadherin mRNA expression levels, concurrently. We also demonstrated that STIP1 knockdown suppressed p-FAK, p-AKT, MMP2, MMP9, and Slug protein and mRNA expression levels, thus, indicating, at least in part, a role for STIP1 in the activation of FAK/AKT/MMP signaling.

Conclusion: Taken together, our results demonstrate a critical role for STIP1 in cancer metastasis, disease progression and poor prognosis, as well as, provide evidence suggestive of the therapeutic efficacy of STIP1-mediated targeting of the FAK/AKT/MMP signaling axis in patients with PANC.

1. Introduction

Pancreatic cancer (PANC), with an incidence of 458,918 and 432,242 deaths in 2018, ranks as the 12th most commonly diagnosed malignancy and 4th major cause of cancer-related deaths in the world [1]. Despite improved diagnostic and advances in multi-faceted therapeutic approaches consisting of radiotherapy, chemotherapy, and surgery, the 5-year survival rate remains low at 8.2%, especially as only a dismal 9% of patients present at early stage when the disease is amenable to surgery and chemotherapy with a 31.5% survivability; however, in reality, patients often present at advanced stage and with no or nonspecific symptoms such as weight loss and abdominal, confounding early diagnosis [2,3]. The insidious course and poor prognosis of pancreatic cancer makes it necessary to develop novel therapeutic approaches including the identification and targeting of disease-driving genes or new therapeutic targets, while elucidating the molecular

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https://doi.org/10.1016/j.prp.2019.152564
Received 29 March 2019; Received in revised form 9 July 2019; Accepted 24 July 2019

mechanisms that underlie the initiation of PANC, its progression and dismal prognosis.

In the last 2 decades, there is increase documentation of the role of stress in tumor initiation and growth; in fact, functional association – causative or resultant, continue to be suggested between stress-induced hormones, neurotransmitters, tumorigenesis, evasion of infiltrating immune cells, production/activation of immunosuppressive molecules, and the evolution of an aggressive tumor microenvironment (TME) [4]. Contextually, the stress-triggered neural signaling mediate the interaction of PANC cells with the complex TME, and is implicated in primary PANC growth, dissemination and progression [5]. Thus, exploring available evidence of the potential involvement of stress-induced immune suppression in the TME, acquisition of a metastatic and drug-resistant phenotype by PANC cells [4–7], the present study investigated the role of one such stress-induced molecule; namely, Stress-induced phosphoprotein 1 (STIP1) in the PANC metastasis, progression, and poor prognosis.

STIP1, also called the heat shock protein (Hsp)70/Hsp90-organizing protein, characterized by its tetratricopeptide repeat (TPR)1, TPR2A, TPR2B motifs and two aspartate/proline-rich (DP1/2) domains, is an adaptor protein chaperone of Hsps, mediating protein transfer between Hsp70 and Hsp90, and subsequently facilitating Hsp70/Hsp90 complex formation, which in itself is essential for the folding and maturation of transcription factors, hormone receptors, and protein kinases [8,9]. Hsps are implicated in the initiation, facilitation and progression of malignancies [10]. While there are reports of STIP1 overexpression in colorectal [11], ovarian [12], cholangiocellular [13], renal [14], breast [15] and gastric [16] cancers, and STIP1$^{-/-}$ mice embryos have been shown to be non-viable, suggesting the critical role of STIP1 in mice embryogenesis and development [17], our understanding of its role in PANC progression and prognosis is evolving, and the underlying molecular mechanisms for its oncogenic activities remain poorly understood.

The present study reports the clinical significance of STIP1 in PANC and shows that STIP1 is essential for the acquisition and maintenance of PANC cell malignant and metastatic properties.

Increased STIP1 expression was positively associated with cancer metastasis and disease progression as well as significantly correlated with decreased overall and disease-free survival. The shRNA-mediated knockdown of STIP1 effectively inhibited PANC cellular migration, invasion, and epithelial-to-mesenchymal transition (EMT). In addition, upon downregulation of STIP1, the expression levels of p-FAK, p-AKT, MMP2, MMP9, and Slug protein were significantly suppressed in human pancreatic cancer cell lines PANC-1 and SW1990. Thus, we show that STIP1 is a promising potential therapeutic target for PANC treatment.

2. Materials and methods

2.1. Patient cohort and clinical samples

PANC and adjacent non-tumor pancreas tissues were obtained from radiation- or chemotherapy-naïve patients (n = 201) who underwent radical pancreatectomy in the Shaoxing People's Hospital (Shaoxing, China) and Tongji Hospital, Tongji University School of Medicine (Shanghai, China) between August 2007 and September 2017. All diagnoses were consistent with the American Joint Committee on Cancer (AJCC) standards. The current study protocol was reviewed and approved by the institutional human research ethics committee and performed in accordance with the Declaration of Helsinki. All tissues were used for tissue array (TMA) constructed by Shangahi Biochip Co., Ltd (Shanghai, China).

2.2. Immunohistochemistry (IHC) analysis

Immunohigtochemical staining was performed using Histostain-Plus IHC Kit (Invitrogen, Waltham, MA) according to the manufacturer's instructions. Briefly, 5-μm sections from the TMAs were baked, deparaffinized and rehydrated; then, treated for antigen retrieving, endogenous peroxidase activity inhibition, and background non-specific staining reduction. After that, the sections were incubated with anti-STIP1 antibody (ab96550, Abcam, Cambridge, UK) at 4 °C overnight, then followed by incubation with biotin-labeled secondary antibody, and HRP-conjugated streptavidin at room temperature for 20 min. Color development was performed with DAB Kit (ZSGB-BIO, Beijing, China). Finally, the sections were counterstained with hematoxylin, dehydrated, cleared, and mounted. Positive and negative controls were processed concurrently. Scoring and interpretation was carried out by 2 independent pathologists.

2.3. Cell lines

The human pancreatic cell lines PANC-1, Capan-2, SW1990 and BxPC-3 were obtained from the American Type Culture Collection (ATCC, Manassas, VA), while the human pancreatic ductal epithelial cells, HPDEC (#T0018001) were purchased from AddexBio (AddexBio Technologies, San Diego, CA). All cells were cultured in Dulbecco's Modified Eagle Medium (DMEM, GIBCO, Carlsbad, CA) supplemented with 10% (v/v) fetal bovine serum (FBS, QuaCell Biotechnology, Zhongshan, China) at 37 °C in humidified atmosphere containing 5% CO_2.

2.4. STIP1 shRNA knockdown

For knockdown of STIP1 in PANC-1 and SW1990 cells, we used the STIP1 human shRNA plasmid kit (Origene, Rockville, MD) according to manufacturer's instruction. Transient transfection was performed by adding 2 μg shSTIP1 plasmids and 5 μl Lipofectamine (Invitrogen, Waltham, MA) into 1×10^4/mL PANC cell suspensions in each well of six-well plates. Stable clones expressing shSTIP1 plasmids via lentivirus as vector were established in the PANC-1 or SW1990 cells. Transfection medium was removed after 24 h and replaced with fresh growth medium. The stably transfected PANC cells were then collected for western blot analysis, migration and invasion assays.

2.5. Wound healing migration assay

For wound healing assay, after wild type or shSTIP1-transfected PANC-1 or SW1990 cells single reached 100% confluence, equal-width scratch-wound lines were made with the tip of sterile 10-μl pipettes. The scratch-wound gaps were monitored, closure recorded, and images taken under microscope at indicated times after the scratch-wound were made.

2.6. Transwell® invasion assays

Invasion assays were performed using the Transwell matrigel system. 1×10^5 wild type or shSTIP1-transfected PANC-1 or SW1990 cells in 100 μl FBS-free DMEM were seeded onto matrigel-coated 8 μm pore Transwell insert (Corning, Corning, NY) placed in Corning 24-well plates containing 500 μl of complete DMEM and incubated at 4 °C for 24 h. After incubation, the cells remaining inside the insert upper surface that did not invade were scraped off with sterile cotton swab. Invaded cells in the lower surface were carefully rinsed with PBS, stained with 0.25% (w/v) crystal violet solution for 10 min, then gently rinsed again with double distilled water and air-dried. The stained inserts were then viewed under microscope and invaded cells per field in 10 random fields were counted at 200× magnification. Experiments were performed in triplicate.

2.7. Western blot analysis

Cells were harvested in lysis buffer (Beyotime, China) involving 1% Complete Mini-Protease Inhibitor Cocktail (Roche, Basel, Switzerland). Lysates were centrifuged at 16,000*g* at 4 °C for 15 min. The BCA Protein Assay Kits (Thermo Fisher Scientific, Waltham, MA) were used to determine protein concentration according to manufacturer's instructions, and protein separated by 10% (w/v) sodium dodecyl sulfate - polyacrylamide gel electrophoresis (SDS-PAGE) under reducing conditions, before blots were transferred onto polyvinylidene difluoride (PVDF) membranes (Millipore, Darmstadt, Germany). The membranes were then blocked with 5% non-fat dried milk in TBS (25 mM Tris–HCl, pH 7.4, 150 mM NaCl, 2.7 mM KCl), and incubated at 4 °C overnight with primary antibodies against STIP1 (Abcam, Cambridge, UK), p-FAK (Abcam), FAK (Abcam), p-AKT (Abcam), AKT (Abcam), MMP2 (Abcam), MMP9 (Abcam), SNAIL + SLUG (ab78105, Abcam), and β-actin (ab6276, Abcam). After overnight incubation, membranes were washed three times with TBS-Tween-20 (1% v/v) for 5 min before incubating with anti-mouse (Santa Cruz Biotechnology, Santa Cruz, CA) or anti-rabbit (Cell Signaling Technology, Danvers, MA) secondary antibodies, for 1 h at RT, followed by TBST washing and band detection using Pierce ECL western blotting substrate (Thermo Fisher Scientific, Waltham, MA). Blot quantification was performed using ImageJ software.

2.8. Reverse transcriptase-PCR analysis

After the RNA extraction from the PANC tissue FFPE samples, for each sample, 1 μg of total RNA was reverse transcribed into cDNA, and the reaction mixture was incubated at 37 °C for 1 h. 1 μl solution was amplified by PCR using primers specific to *STIP1* and *GADPH*. STIP1: forward – 5′-ACTAACATGACTTACATTACC-3′; reverse, 5′-ATATGCTT TGGCAATCTG-3′. GAPDH: forward – 5′-AATCCCATCACCATCTT CCA-3′; reverse – 5′-TGGACTC CACGACGTACTCA-3′. The PCR mixture consisted of 10 mM Tris–HCl (pH 8.3), 50 mM KCl, 0.25 mM deoxynucleotide triphosphate (dNTP), 1.5 mM $MgCl_2$, 2 units of AmpliTaq Gold DNA polymerase (Thermo Fisher Scientific Inc. Waltham, MA), and 0.5 μM of each primer. PCR was performed at a final volume of 50 μl. After incubation at 95 °C for 3 min, STIP1 mRNA was amplified for 35 cycles at 95 °C for 1 min, 60 °C for 1 min, and 72 °C for 1 min 30 s, and GADPH was amplified for 20 cycles using same condition, but followed by extension phase at 72 °C for 10 min. For quantification and visualization of RT-PCR product, 10 μl of each PANC sample was subjected to electrophoresis using the 12% polyacrylamide gel, stained with 0.2% ethidium bromide, and the intensity of every band was then evaluated under UV light using the CCD image sensor.

2.9. Statistical analysis

All data is representative of experiments performed at least 3 times in triplicates and are expressed as mean ± SD. SPSS 25.0 software (Version 25.0. Armonk, NY) was used for data analyses. Student's *t*-test was used for comparing statistical difference between two groups. Kaplan-Meier curves and multi-group significance was compared using the one-way analysis of variance (ANOVA) followed by the Dunnett's post hoc test. P-value < 0.05 was considered statistically significant.

3. Results

3.1. High expression of STIP1 positively correlates with disease progression in patients with PANC

To investigate the clinical relevance of STIP1 in PANC, STIP1 expression levels in patient tumor tissues were evaluated by IHC staining. As shown in Fig. 1A, high STIP1 expression levels were observed in PANC tissues compared with no expression in the adjacent normal pancreatic tissue ($p < 0.01$) or mild pancreatic intraepithelial neoplasia tissues ($p < 0.05$). Based on the STIP1 staining intensity, our cohort was divided into STIP1 low (n = 90) characterized by negative or weak STIP1 staining and STIP1 high (n = 111) characterized by moderate or strong STIP1 staining (Table 1). The associations between the expression of STIPI and the clinicopathological characteristics of our PANC cohort, including patients' age, gender, tumor size, TNM stage, cancer metastasis, microvascular invasion, perineural invasion (PNI), carcinoembryonic antigen (CEA), and vital status are summarized in Table 1. Clinicopathological correlation studies revealed significant positive correlation between high STIP1 expression and lymph node involvement ($p = 0.001$), cancer metastasis ($p = 0.002$), microvascular invasion ($p = 0.002$), advance TNM stage ($p = 0.024$), perineural invasion (PNI; $p = 0.013$), and cancer-related death ($p = 0.002$) among patients with PANC (Table 1). These results suggest that STIP1 overexpression is significantly associated with PANC progression.

3.2. Elevated expression of STIP1 is strongly associated with poor prognosis in patients with PANC

To determine if and to what extent STIP1 expression levels affect and/or predict patient survival after surgical resection or pancreatectomy, using a randomly selected subset of the total cohort (n = 88; 43.8% of total cohort) (Fig. 1B), survival data of patients with STIP1 low (n = 42; 46.7% of all STIP1 low) and STIP1 high (n = 46; 41.4% of all STIP1 high) based on median STIP1 expression cut-off were analyzed and Kaplan-Meier (KM) curves plotted (Table 1). Compared to patients with STIPI low expression, the overall survival (OS: $p < 0.001$) and disease-free survival (DFS: $p = 0.001$) of patients with STIP1 high expression was significantly worse (Table 2 and Table 1). Our univariate analysis of factors associated with patient survival and disease recurrence showed that, positive smoking status, higher smoking pack-year, alcohol consumption, cancer metastasis, tumor size ≥ 5 cm, multiple tumor type, vascular invasion, advanced TMN stage, and high STIP1 expression levels are positively correlated with poor OS and DFS in patients with PANC (Table 2). Furthermore, multivariate analysis indicate that high STIPI expression (HR = 2.11; 95% CI = 1.76–3.42; $p < 0.001$), Cancer metastasis (HR = 1.51; 95% CI = 1.22–1.74; $p = 0.002$), tumor size ≥ 5 cm (HR = 1.25; 95% CI = 1.14–2.15; $p < 0.001$), advanced TNM stage (HR = 1.12; 95% CI = 1.21–1.86; $p < 0.001$), and positive smoking status (HR = 2.12; 95% CI = 1.72–3.25; $p < 0.001$) were independent prognostic factors for overall survival (Table 2). As indicated above, disease-free survival was significantly shorter in the STIPI high group compared to the STIP1 low expression patients ($p = 0.001$; Fig. 1C). This association was confirmed by univariate ($p = 0.001$) and multivariate analysis (HR = 2.71; 95% CI = 1.08–4.21; $p = 0.001$) (Table 2). These results indicate that STIP1 overexpression is associated with poor prognosis and is an independent prognostic factor in patients with PANC.

3.3. STIP1 knockdown significantly inhibit the invasive and metastatic traits of PANC-1 and SW1990 cells

There is evidence that cancer metastasis is strongly associated with the poor prognosis in patients with PANC [18]; thus, having observed a positive correlation between STIP1 expression and poor prognosis, we investigated the likely effect of STIP1 expression on PANC cell migration and invasion. Firstly, we confirmed that compared to no or very mild expression in the human pancreatic ductal epithelial cells (HPDECs), STIP1 expression was significantly upregulated in human PANC cell lines PANC-1, Capan-2, SW1990 and BxPC-3, both at protein (Fig. 2A) and mRNA levels (Fig. 2B). Secondly, since strong STIP1 expression was most apparent in PANC-1 and SW1990 cells, using STIP1-specific shRNA, we significantly suppressed the expression of STIP1 in PANC-1 and SW1990 cells both at mRNA (PANC-1, $p < 0.01$; SW1990, $p < 0.01$; Fig. 2C) and protein levels (Fig. 2D). Results of our western

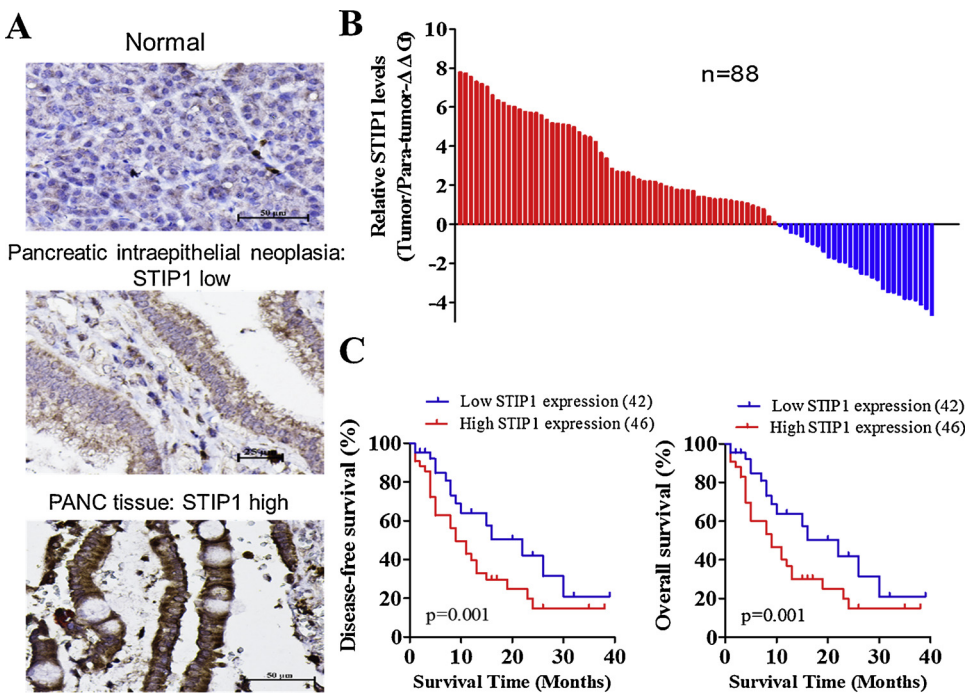

Fig. 1. High expression of STIP1 positively correlates with disease progression and poor prognosis in patients with PANC. (A) Representative IHC images of STIP1 expression in normal pancreatic (upper panel), pancreatic intraepithelial neoplasia (middle panel) and PANC tissue samples. (B) Graphical representation of the relative STIP1 expression levels in PANC tissues compared with adjacent non-tumor tissues. (C) High STIP1 expression was associated with the worse overall (left panel) and disease-free (right panel) survival as shown by KM curves. KM curves, Kaplan-Meier curves.

Table 1
Clinicopathological correlations of STIP1 expressions in pancreatic cancer patients determined by IHC.

Clinical parameters		number	expression		*P* value
			Low	High	
Age (years)					
	< 55	131	62	69	0.209
	≥55	70	28	42	
Gender					
	Male	164	67	97	0.503
	Female	38	17	21	
Size					
	< 5	40	13	27	0.172
	≥5	158	75	83	
Lymphatic metastasis					
	M0	28	14	14	**0.001**
	M1	166	73	93	
Cancer metastasis					
	M0	125	63	62	**0.002**
	M1	70	25	45	
Tumor type					
	solitary	10	7	3	0.172
	multiple	181	77	104	
Microvascular invasion					
	Absence	115	47	68	**0.002**
	Presence	46	17	29	
TNM stage					
	I-II	37	12	25	0.024
	III	145	63	82	
PNI					
	Negative	102	45	57	**0.013**
	Positive	68	28	40	
CEA					
	< 5	121	51	70	0.132
	≥5	1	0	1	
Status	Dead	100	53	47	**0.002**
	Alive	64	20	44	

Abbreviation: PNI, perineural invasion; CEA, carcinoembryonic antigen.

blot analysis showed this suppression of STIP1 expression was strongly associated with reduced migration in PANC-1 ($p < 0.01$) and SW1990 ($p < 0.01$) cells (Fig. 3A and B). At same time, Matrigel invasion assay showed that suppressed STIP1 expression significantly inhibited the number of invaded PANC cells ($p < 0.001$; Fig. 3C and D) and was associated with concurrent upregulated E-cadherin, and downregulated N-cadherin and vimentin mRNA expression levels (Fig. 3E). These results indicate that STIP1 knockdown significantly inhibit the migration and invasive ability of PANC-1 and SW1990 cells, as well as deter EMT.

3.4. STIP1-mediated inhibition of EMT and anti-metastasis effect in PANC cells is through the deactivation of FAK/AKT/MMP signaling axis

Understanding that the interaction with and between integrins and focal adhesion kinase (FAK) regulates the adhesion of malignant cells and their subsequent invasion into the extracellular matrix; especially as the phosphorylation (activation) of FAK and its downstream targets correlate with increased cellular motility and invasion [19], to determine the molecular mechanism underlying the EMT-inhibiting and anti-metastasis effect STIP1 knockdown, we comparatively examined the involvement of the molecular expression of the FAK/AKT signaling pathway and associated regulators of metastasis in STIP1-knockdown (shSTIP1), shRNA control (shControl) and wild-type negative control (NC) SW1990 cells. Our results demonstrated that STIP1 knockdown concurrently suppressed p-FAK, p-AKT, matrix metalloproteinase (MMP)2, MMP9, and slug expression at protein (Fig. 4A) and mRNA (Fig. 4B) levels, thus, indicating, at least in part, a role for STIP1 in the activation of FAK/AKT/MMP signaling pathway.

4. Discussion

The present study reports the significant upregulation of STIP1 expression in PANC tissues and its correlated with was correlated with the metastatic traits, disease progression, and poor prognosis in patients with PANC (Fig. 1). Our findings indicate a critical role for STIP1 in the facilitation of cancer-promoting EMT. Our study provides evidence that the silencing of STIP1 significantly attenuates the migration, invasion and metastatic phenotype of PANC cells, as well as inhibits their EMT. As alluded above, enhanced expression of STIP1 has been documented in several malignancies, indicating an oncogenic role for the upregulation of STIP1, and that it might be characteristic of malignancies and malignant processes (Figs. 2 and 3).

These findings are consistent with those from a recent meta-analysis

Table 2
Univariate and multivariate analyses of factors associated with survival and recurrence.

Parameters	Univariate	Overall survival Multivariate		Univariate	Disease free survival Multivariate	
	p	HR (95% CI)	*p*	*p*	HR (95% CI)	*p*
Age (< 55 vs. ≧55)	0.459			0.35		
Gender (male vs. female)	0.132			0.02		
Smoking status (yes vs no)	< 0.001	2.12(1.72–3.25)	< 0.001	0.01	1.31(1.32–2.66)	0.025
Smoking pack –years (light vs heavy)	0.001	2.03(1.51–3.01)	0.002	0.001	1.5(1.51–2.05)	0.023
Alcohol consumption (low vs high)	0.009	1.14(1.01–2.62)	0.01	0.001	1.6(1.51–2.32)	0.002
CEA (< 5 vs. ≧5 ng/mL)	0.92			0.65		
Viral status (yes vs. no)	0.13			0.34		
Cancer metastasis (yes vs no)	0.02	1.51(1.22–1.74)	0.002	0.001	1.12(1.14–2.62)	
Tumor size (< 5 cm vs. ≧5 cm)	0.04	1.25(1.14–2.15)	< 0.001	0.001	1.08(1.73–2.05)	0.028
Tumor type (solitary vs. multiple)	< 0.001	1.72(1.74–2.13)	0.05	0.001	1.03(1.12–2.61)	< 0.001
Vascular Invasion s (yes vs. no)	0.008	1.62(1.35–2.13)	0.002	0.01	2.16(1.32–3.84)	0.008
TNM stage (I/II vs III/IV)	0.01	1.12(1.21–1.86)	< 0.001	0.01	1.23(1.81–2.01)	0.012
STIP1 (low vs high)	< 0.001	2.11(1.76–3.42)	< 0.001	0.001	2.71(1.08–4.21)	0.001

CEA carcinoembryonic antigen, Smoking pack –years; Light smoker (PY > 0 and < 20), Heavy smoker (PY ≧ 20), Alcohol consumption: low-moderate (1–50 g/day), medium-high drinker (> 50 g/day).

which explored the prognostic value of STIP1 expression in malignancies from 9 studies with a total of 1417 patients with cancer; results of this meta-analysis showed a 0.5 prevalence of high STIP1 expression in patients with cancer, with the high expression of STIP1 being significantly associated with lymph node metastasis ($p < 0.01$) and advanced clinical stage ($p < 0.01$), and predictive of shorter overall (HR = 1.40, $p < 0.01$) and disease-free (HR = 1.30, $p < 0.01$) survival [20]. In fact, consistent with recent findings by Zhang, et al. [21] reporting that the aberrant expression of STIP1 in colorectal cancer was significantly correlated with advance TNM stage and is an independent prognostic factor in patients with colorectal cancer, we also demonstrated significant positive correlation between high STIP1 expression and lymph node involvement ($p = 0.001$), cancer metastasis ($p = 0.002$), microvascular invasion ($p = 0.002$), advance TNM stage ($p = 0.024$), and cancer-related death ($p = 0.002$) among patients with PANC (Table 1), suggesting that STIP1 overexpression is significantly associated with PANC progression. Our clinicopathological correlation findings are of relevance, especially as PNI, which is characteristic of a broad spectrum of malignancies, is a reliable indicator of cancer invasion and metastasis, and is a predictor of poor prognosis in patients with cancer [22].

Previously thought to occur as a result of low-resistance channels in the anatomic region of interest, it is now understood that PNI results from malignant alterations in tumoral nerve cells and supporting cells namely astrocytes, oligodendrocytes, microglial, and ependymal cells in the tumor microenvironment; architectural re-arrangement and cellular migration of the perineural matrix; enhanced viability and invasiveness of cancer cells; regeneration of nerve cells, and their loss of adherence similar to their neighboring cancer cells; and the increased evasion of immunological surveillance, autophagy, and apoptosis of cancer cells [22]. This is indicative of a putative role for STIP1 as not only a marker of oncogenicity and malignant progression in PANC, but as a prognosis factor in patient with PANC. Thus, it only scientifically logical that STIP1 knockdown significantly inhibits the invasive and metastatic

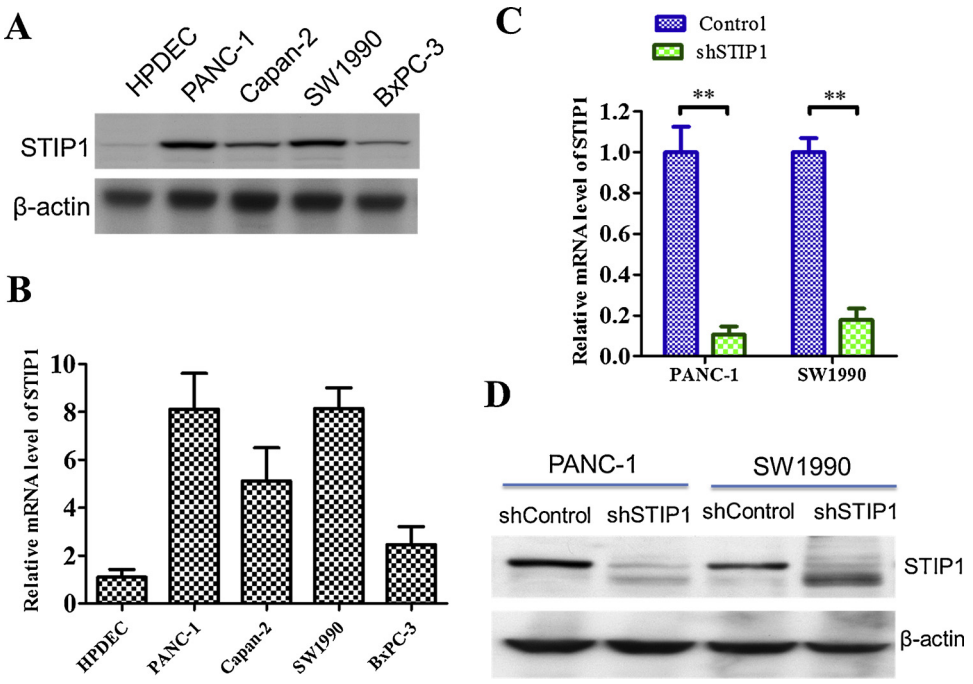

Fig. 2. STIP1 is highly expressed in human PANC cells compared to non-tumor human pancreatic duct epithelial cells (HPDEC). Western blot images showing STIP1 expression significantly upregulated in human PANC cell lines PANC-1, Capan-2, SW1990 and BxPC-3, both at (A) protein and (B) mRNA levels, compared to HPDEC. (C) RT-PCR and (D) western blot analysis show significant knockdown efficacy of shSTIP1 in PANC-1 and SW1990 cells. β-actin served as loading control. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$.

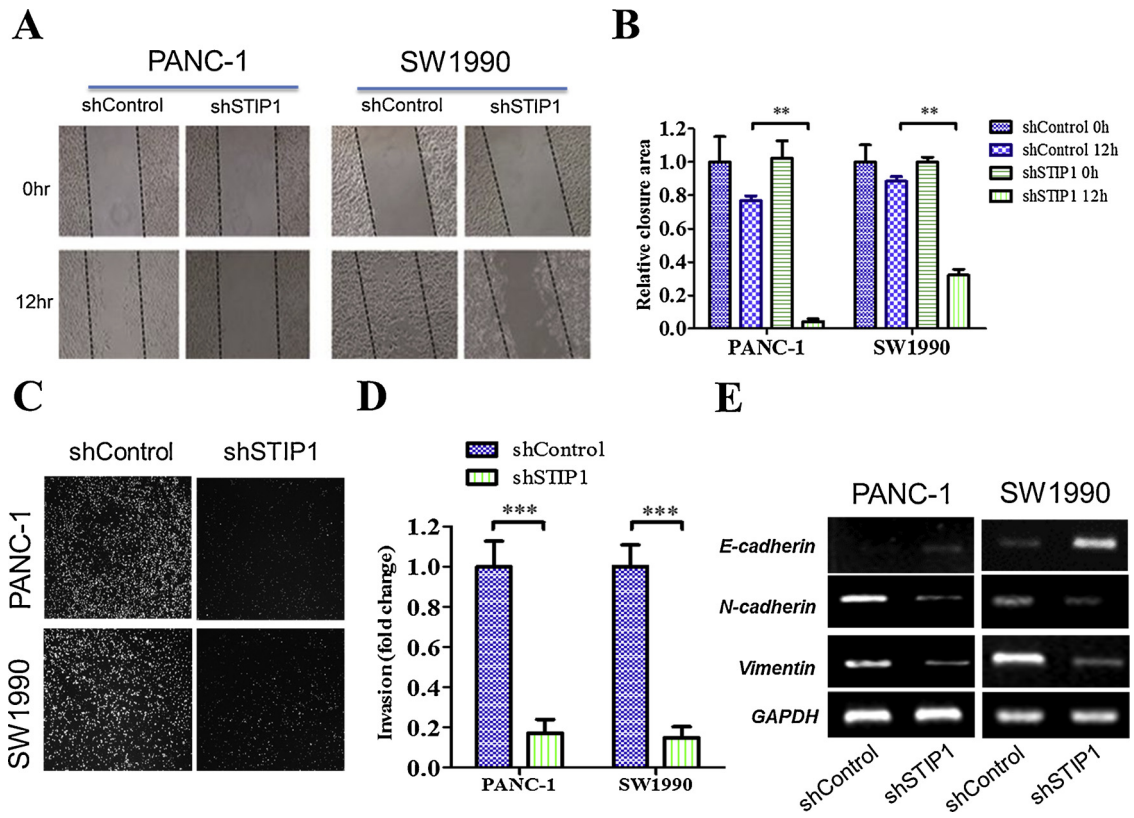

Fig. 3. STIP1 knockdown significantly inhibit the invasive and metastatic traits of PANC-1 and SW1990 cells. (A) Image and (B) histograms showing that shSTIP1 significantly decreased the migration of PANC-1 and SW1990 as shown by wound healing assay. (C) Image and (D) histograms showing the inhibitory effect of shSTIP1 on the invasion of PANC-1 and SW1990. (E) Representative RT-PCR data showing reduced N-cadherin and vimentin, but upregulated E-cadherin mRNA levels in shSTIP1 PANC-1 and SW1990 cells, compared to the shControl cells. $*p < 0.05$, $**p < 0.01$, $***p < 0.001$.

traits of PANC-1 and SW1990 cells as we demonstrated (Figs. 2 and 3).

Furthermore, since the interaction between integrins and FAK have been shown to regulate the adhesion of malignant cells and their subsequent invasion into the extracellular matrix; especially as the activation of FAK and its downstream targets correlate with increased cellular motility and invasion [19], we provided evidence suggesting that the deactivation of the FAK/AKT signaling pathway and associated regulators of metastasis underlie the shown EMT-inhibiting and anti-metastasis effect STIP1 knockdown, as shown by concurrently suppressed p-FAK, p-AKT, MMP2, MMP9, and slug expression at protein and mRNA levels, thus, indicating, at least in part, a role for STIP1 in the activation of FAK/AKT/MMP signaling pathway (Fig. 4). These findings are in accordance with findings showing that ectopic expression of FAK in Jurkat T and primary T cells induced fibronectin (FN)-mediated increase in the production of MMP2 and MMP9 [23]. Initiation factors, such as slug are known to initiate and facilitate EMT by acting on effector molecules, including the proteinases MMP2 and MMP9 which actively induce extracellular matrix (ECM) degradation, and cancer cell migration and invasion, and are often dysregulated in cancer progression [24]. This is consistent with results we have

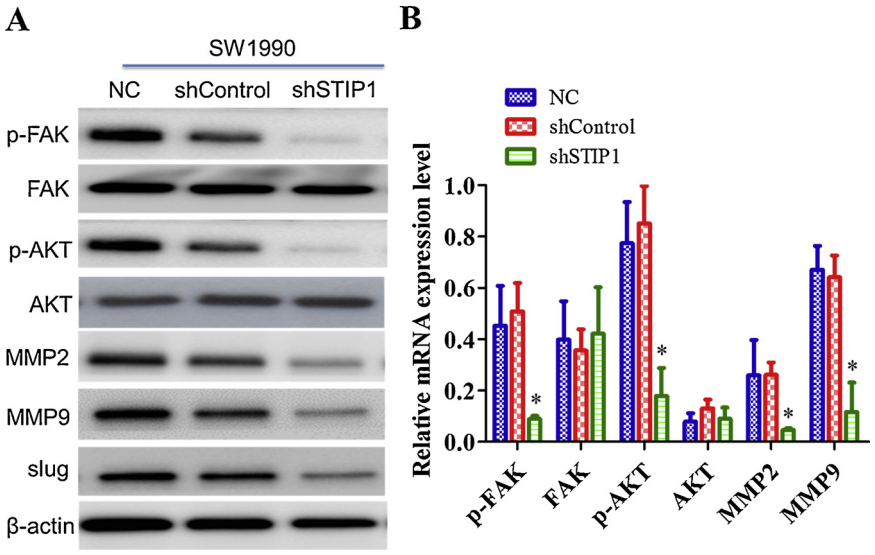

Fig. 4. STIP1-mediated inhibition of EMT and anti-metastasis effect in PANC cells is through the deactivation of FAK/AKT/MMP signaling axis. Representative (A) western blot analysis images and (B) graphical representation of RT-PCR data showing the effect of shSTIP1 on the expression levels of p-FAK, FAK, p-AKT, AKT, MMP2, MMP9, and slug in shSTIP1 SW1990 cells, at protein and mRNA level, respectively, compared to their NC or shControl counterparts.NC, negative control; $*p < 0.05$, $**p < 0.01$, $***p < 0.001$.

presented, and validate the proposition that alterations in the expression of STIP1 do regulate ECM degradation, EMT, cancer cell motility, and cancer progression through the deactivation of FAK/AKT/MMP signaling, and is consistent with the work of Kwiatkowska, et al. suggesting that the de-phosphorylation of AKT and FAK suppress glioblastoma cell invasion by downregulating the expression of MMPs and impairing the shuttling of membrane type 1 metalloproteinase (MT1-MMP) to the lamellipodia [25].

While further studies are required to elucidate on the molecular target of STIP1 in PANC cells, the results of this present study indicating that STIP1 actively regulate the malignant and metastatic phenotypes of PANC cells by modulation of the FAK/AKT/MMP pathway and is a potential molecular target (Fig. 4), is in line with those observed by Chen et al. [26] suggesting that the induction of cell migration and invasion by the Sonic hedgehog signaling pathway is through FAK/AKT signaling-mediated activation of MMP2 and MMP9 in liver cancer. This is similarly corroborated in studies on colorectal cancer [27] showing that FAK/AKT/MMPs signaling axis mediates the induction of EMT and subsequently promote metastasis of colorectal cancer.

In conclusion, we present data indicating that high STIP1 expression in PANC tissues is positively associated with poor survival in patients with PANC, and that when STIP1 is knocked down, it results in the loss of malignant and metastatic phenotypes of PANC cells through deactivation of FAK/AKT/MMP signaling pathway. Taken together, our results demonstrate a critical role for STIP1 in cancer metastasis, disease progression and poor prognosis, as well as, provide evidence suggestive of the therapeutic efficacy of STIP1-mediated targeting of the FAK/AKT/MMP signaling axis in patients with PANC.

Authors' contributions

Conceived and designed the study: Yuanming Jing, Wenqing Liang, Performed the experiments: Jian Liu, Lin Zhang, Jianguo Wei, Provided reagents, materials, experimental infrastructure and administrative oversight: Yafang Zhu, Jianhui Yang, Kewei Ji. Wrote the manuscript: Yu zhang, Zongliang Huang. All authors read and approved the final version of the manuscript.

Disclosure statement

The authors have declared no conflict of interest.

Acknowledgments

This study was supported by Public Welfare Application Plan Project of Shaoxing (2018C30109), Nature Science Foundation of Zhejiang Province (LY18H060013), Project of Health Commission of Zhejiang province (2018KY831).

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