Cancer Letters 451 (2019) 48–57

Contents lists available at ScienceDirect

Cancer Letters

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

Original Articles

WT1 associated protein promotes metastasis and chemo-resistance to gemcitabine by stabilizing Fak mRNA in pancreatic cancer

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ARTICLE INFO

Keywords:
WTAP
Fak
PC
Migration/invasion
Chemo-sensitivity

ABSTRACT

WT1 associated protein (WTAP), playing an important role in several malignancies owing to its complex function in transcriptional and post-transcriptional regulation, is an independent prognostic indicator for pancreatic cancer (PC). However, its specific role and underlying mechanism in PC remain unclear. In the present study, we found that WTAP could promote migration/invasion and suppress chemo-sensitivity to gemcitabine in PC. Further mechanical investigation revealed that WTAP could bind to and stabilize Fak mRNA which in turn activated the Fak-PI3K-AKT and Fak-Src-GRB2-Erk1/2 signaling pathways. In addition, GSK2256098, a specific Fak inhibitor, could reverse WTAP-mediated chemo-resistance to gemcitabine and metastasis in PC. Taken together, Fak inhibitor might be a promising therapeutic option for PC patients with WTAP overexpression.

1. Introduction

Pancreatic cancer (PC) has a mortality rate which closely parallels the corresponding incidence rate [1] making it the most lethal malignancy among digestive tract tumors. The American Cancer Society estimates that PC will be the fourth leading cause of cancer death in the USA by the end of 2018 with approximately 55440 newly diagnosed cases and nearly 45000 related deaths in 2018 [2]. Similar data from the National Central Cancer Registry of China suggested the incidence and related mortality rates of PC rapidly increased between 2000 and 2011 [3], meaning PC is a global health issue which is causing a significant medical burden around the world. Radical resection followed by adjuvant therapy remains the standard treatment for PC. However, due to its early recurrence/metastasis and resistance to chemotherapy, the 5-year survival is only up to 25% even after receiving potential curative resection [4]. Moreover, even the newly developed immunotherapies, including anti-programmed death 1 or programmed death 1 ligand 1 (PD-1/PDL-1) treatment, showed a poor effect on PC [5]. Therefore, detailed investigation of the molecular mechanisms of tumor cell metastasis and chemo-resistance to gemcitabine in PC is necessary and may provide insight for new treatment targets and evidence to improve PC prognosis.

WT1 associated protein (WTAP), a nuclear protein, is first identified by Little [6] who noticed its specific interaction with WT1. Besides several essential physiological processes, such as mRNA stabilization [7], eye development [8], m6A methylation [9], mRNA alternative splicing [10] and cell cycle regulation [11], WTAP is also involved in the carcinogenesis and progression of several malignant tumors, including glioblastoma [12], cholangiocarcinoma [13], acute myeloid leukemia (AML) [14], colorectal cancer (CRC) [15] and renal cell carcinoma (RCC) [16]. In our previous study, WTAP was identified as an independent prognostic indicator for PC, whose high expression in the nucleus had a significant relationship with advanced N stage and dismal prognosis [17]. It has been demonstrated that WTAP plays an oncogenic role by targeting WT1-TBL1 axis in CRC [15], while by stabilizing CDK2 mRNA in RCC [16]. However, the exact molecular mechanism in PC is still not unveiled.

Focal adhesion kinase (Fak) is a cytoplasmic protein tyrosine kinase that is overexpressed and activated in several solid cancers [18]. Increased Fak dimerization induced by higher Fak levels contributes to its

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https://doi.org/10.1016/j.canlet.2019.02.043
Received 7 December 2018; Received in revised form 14 February 2019; Accepted 28 February 2019

catalytic activation [19]. Its downstream signaling pathways are extremely complex and vary upon different tumor types, including Fak-RHOGEF-RHO signaling pathway; Fak-PI3K-AKT signaling pathway; Fak-Src-GRB2-Erk1/2 signaling pathway; Fak-JNK-JUN signaling pathway et al. [18]. Several studies also confirmed a strong relationship between Fak and PC. Firstly, Fak expression correlated significantly with the T and M stages in PC [20,21]. Secondly, activation of Fak was involved with the aggressive capability in PC [22], while Fak silencing could reverse its pro-metastatic effect [23]. Thirdly, Fak could increase intrinsic chemo-resistance to gemcitabine in PC [24], whereas RNA interference [25] or small molecular inhibitor [26,27] targeting Fak could enhance chemo-sensitivity to gemcitabine. Here, we provided the first evidence that overexpression of WTAP increased cell mobility and chemo-resistance to gemcitabine in both MIA PaCa-2 and BxPC-3 cells, while down-regulation of WTAP had an opposite effect. The positive correlation between WTAP and Fak expression was further confirmed in PC tissues by using GEPIA. Mechanically, WTAP could stabilize Fak mRNA and activate Fak signaling pathway. Moreover, a small molecular Fak inhibitor, GSK2256098, could reverse WTAP-induced chemo-resistance and metastasis which indicated that Fak might be a promising therapeutic target for PC.

2. Materials and method

2.1. Cell culture

Human PC cell lines, including MIA PaCa-2, BxPC-3, T3M4, PANC-1 and AsPC-1, and normal pancreatic duct epithelial cell lines, including HPDE6-C7 and hTERT-HPNE, were obtained from the American Type Culture Collection (ATCC, USA) and routinely cultured in DMEM or RPMI1640 supplemented with 10% fetal bovine serum (FBS) (HyClone, USA). Cells were incubated in a humidified atmosphere with 5% CO_2 at 37 °C.

2.2. Transfection

MIA PaCa-2 and BxPC-3 were seeded in twelve-well plates and cultured for 24 h. When the confluence reached at 50%, the cells were infected with WTAP overexpression lentivirus (termed as WTAP-OE), negative control lentivirus (termed as WTAP-NC), WTAP knockdown lentivirus (termed as shWTAP), and scramble control lentivirus (termed as shNC) according to the manufacturer's instructions. Lentivirus constructing of WTAP overexpression or knockdown were purchased from Gene-Pharma (Shanghai, China). Stable transductions were selected using puromycin (1–2 μg/ml) for 2 weeks. Mono-clone isolation was conducted following the protocol provided by CORNING (USA). The specificity and efficiency were validated by western blot and qRT-PCR in the stable cell lines.

2.3. Protein isolation and western blot assay

Whole cell proteins were extracted using RIPA buffer containing protease inhibitors (Sigma, USA). The BCA Protein Assay kit (Pierce, USA) was used to quantify the protein concentration. About 50 μg protein was separated by 10% or 12% SDS-PAGE gel and transferred onto a PVDF membrane (Millipore, USA). After blocking, membranes were incubated with the following primary antibodies: anti-WTAP antibody (Abcam, USA), anti-Fak antibody (Cell Signaling Technology, USA), anti-p-Fak antibody (Cell Signaling Technology, USA), anti-Src antibody (Cell Signaling Technology, USA), anti-p-Src antibody (Cell Signaling Technology, USA), anti-AKT antibody (Cell Signaling Technology, USA), anti-p-AKT antibody (Cell Signaling Technology, USA), anti-Erk1/2 antibody (Cell Signaling Technology, USA), anti-p-Erk1/2 antibody (Cell Signaling Technology, USA), anti-β-actin antibody (Cell Signaling Technology, USA) and anti-GAPDH antibody (Santa Cruz Biotechnology, USA) (Supplementary file. 1). The membranes were then incubated with HRP-conjugated secondary antibodies at room temperature (Applygen Technologies Inc., Beijing, China). After washes, the protein bands were visualized using enhanced chemiluminescence detection reagents (Applygen Technologies Inc., Beijing, China).

2.4. RNA isolation and qRT-PCR

Total RNAs were extracted from cultured cell lines by Trizol reagent (Invitrogen, USA). Then cDNA was synthesized using the TaqMan Reverse Transcription Kit (Takara, Dalian, China) according to the manufacturer's instructions. For WTAP and Fak mRNA analysis, qRT-PCR was performed with SYBR® Premix Ex TaqTM Reagent (TaKaRa, Dalian, China) by using StepOne Plus Real-Time PCR system (Applied Biosystems, USA) (Supplementary file. 1). Fold changes relative to β-actin were calculated using $2^{-\Delta\Delta Ct}$ method.

2.5. Transwell cell migration and invasion assay

In this assay, about 1×10^4 PC cells were seeded into the upper chambers coated with or without Matrigel (BD Biosciences, USA) for the invasion and migration assays. Medium containing 10% FBS was added to the lower chambers as a chemoattractant. In inhibitor experiment, GSK2256098 (Selleck, USA) was added into the medium at the concentration of 2/5 μM. After incubation at 37 °C for 48 h, the non-invading cells were gently removed with a cotton swab, while the invasive cells located on the lower surface of upper chambers were fixed in methanol for 20 min and stained with hematoxylin and eosin (H&E) for 15 min and 6 min respectively. The invasive cells were counted by an inverted microscope from five random fields and experiment was repeated three times.

2.6. Cytotoxicity assay

For cytotoxicity assay, 4×10^3 PC cells were seeded into 96-well plates. After 6 h, attached cells were treated with various doses of gemcitabine (0 nM, 100 nM, 1 μM, 10 μM, 100 μM, 1 mM) for 48 h. In inhibitor experiment, GSK2256098 (Selleck, USA) was also added into the wells at the concentration of 2/5 μM after cell attachment. CCK-8 kit (Dojindo, Japan) was used to detect cell viability according to the manufacturer's instructions. The inhibition rate was calculated as follows: OD = OD450-OD630, inhibition rate = $1-(OD_{GEM}-OD_{Blank})/(OD_{PBS}-OD_{Blank})$.

2.7. Cell apoptosis assay

PC cells were seeded into twelve-well plates and gemcitabine (LILLY, France) was added into the wells at the concentration of 10 μM after the confluence reached at 70%–80%. In inhibitor experiment, GSK2256098 (Selleck, USA) was also added into the wells at the concentration of 2/5 μM. After incubation at 37 °C for 48 h, cells were collected for apoptosis analysis using an Annexin V-FITC/PI apoptosis assay kit (NeoBioscience, China). Briefly, cells were stained with Annexin V-FITC for 10 min and PI for 5 min at room temperature in the dark. Then the cells were analyzed immediately by AccuriC6 flow cytometer (BD Biosciences, USA) and the primary data was analyzed with Flowjo software (Tree Star, Inc.).

2.8. Fak mRNA stability assay

PC cells transfected with the WTAP-OE, WTAP-NC, shWTAP or shNC lentivirus were treated with 10 μg/mL actinomycin D (MCE, USA) for 0, 2, 4, 6 h. Total RNAs were harvested and then the Fak and β-actin mRNA levels were determined by qRT-PCR. The relative percentage of remaining Fak mRNA was calculated after normalizing to that of β-actin.

2.9. RNA immunoprecipitation

RNA immunoprecipitation (RIP) experiment was performed using Magna RIP RNA-Binding Protein Immunoprecipitation Kit (Millipore, USA) according to the manufacturer's instructions. Briefly, PC cells were lysed by RIP lysis buffer and then cell lysates were immunoprecipitated with protein A/G magnetic beads conjugated to anti-WTAP anti-body (Abcam, USA) or normal rabbit IgG at 4 °C overnight. After RNA purification, qRT- PCR was used to measure the levels of Fak transcript in the protein-RNA complexes.

2.10. GSK2256098 concentration selection

PC cells transfected by WTAP-NC or WTAP-OE lentivirus were seeded into 6-well plates and cultured by either standard medium (WTAP-NC) or medium containing various amount of GSK2256098 (Selleck, USA) (0–10 μM) (WTAP-OE) respectively. After incubation at 37 °C for 48 h, Fak and p-Fak expression level were detected by western blot. The GSK2256098 (Selleck, USA) concentration, at which the WTAP-OE group had a similar or slightly lower p-Fak level than the WTAP-NC group, was selected for the following inhibitor experiment.

2.11. PC orthotopic xenograft model

Animal studies were approved by the Animal Research Ethics Committee of Peking Union Medical College Hospital. Twenty-four female BALB/c athymic nude mice, which were 4–6 weeks old and weighed 20.0–25.0 g, were obtained from the Animal Research Center of PUMCH. WTAP-OE, WTAP-NC, shWTAP and shNC-lentivirus infected MIA PaCa-2 cells (5×10^6) were suspended in 50 μl PBS and then injected subcapsularly into the pancreatic tissue by 1-mL syringes. To prevent leakage of the cells, a cotton wool tip was pressed onto the injection site for 30 s. Six nude mice were included in each group. Eight weeks later, mice were sacrificed and the tumor volume, tumor weight, distal metastasis and spleen infiltration were assessed. Tumor volume was monitored by measuring the length and width ($V = (L \times W^2) \times 0.52$).

2.12. Histologic and immunohistochemical assay

The PC tumors were separated from the pancreatic tissues of the orthotopic xenograft model and fixed in formalin for one day. Paraffin embedding, sectioning and staining with hematoxylin-eosin were performed. Immunohistochemical analyses were conducted according to standard procedures. Briefly, after deparaffinization, rehydration, antigen retrieval and endogenous peroxidase blockage, sections were incubated with anti-WTAP antibody (1:100 dilution, Abcam, USA) at 4 °C overnight. Subsequently, sections were washed by PBS and incubated with HRP-labeled secondary antibody for 30 min. After application of diaminobenzidine as a chromogen, the slides were evaluated using light microscopy (Olympus, Japan).

2.13. Statistical analysis

GraphPad Prism 7 (GraphPad Prism version 7.0, Inc., La Jolla, USA) was used to conduct the data analysis. Each experiment was repeated three times and the results were presented as means ± SD. The differences between groups were analyzed by Student's *t*-test. A P value of < 0.05 was considered as statistically significant.

3. Results

3.1. MIA PaCa-2 and BxPC-3 were selected for further investigation

WTAP and Fak expression were explored in five PC cell lines (MIA PaCa-2, BxPC-3, T3M4, PANC-1 and AsPC-1) and two normal pancreatic duct epithelial cell lines (HPDE6-C7 and hTERT-HPNE) using western blot and qRT-PCR. We found that PANC-1 had the highest WTAP expression level whereas HPDE6-C7 had the lowest level. MIA PaCa-2 and BxPC-3 had an intermediate WTAP expression level (Fig. 1A). The qRT-PCR showed that WTAP mRNA expression corresponded to its protein expression (Fig. 1A). According to the above results, MIA PaCa-2 and BxPC-3 were selected for further investigation. As described in the methods section, the mono-clonal stable transductions of both WATP overexpression and knockdown were established

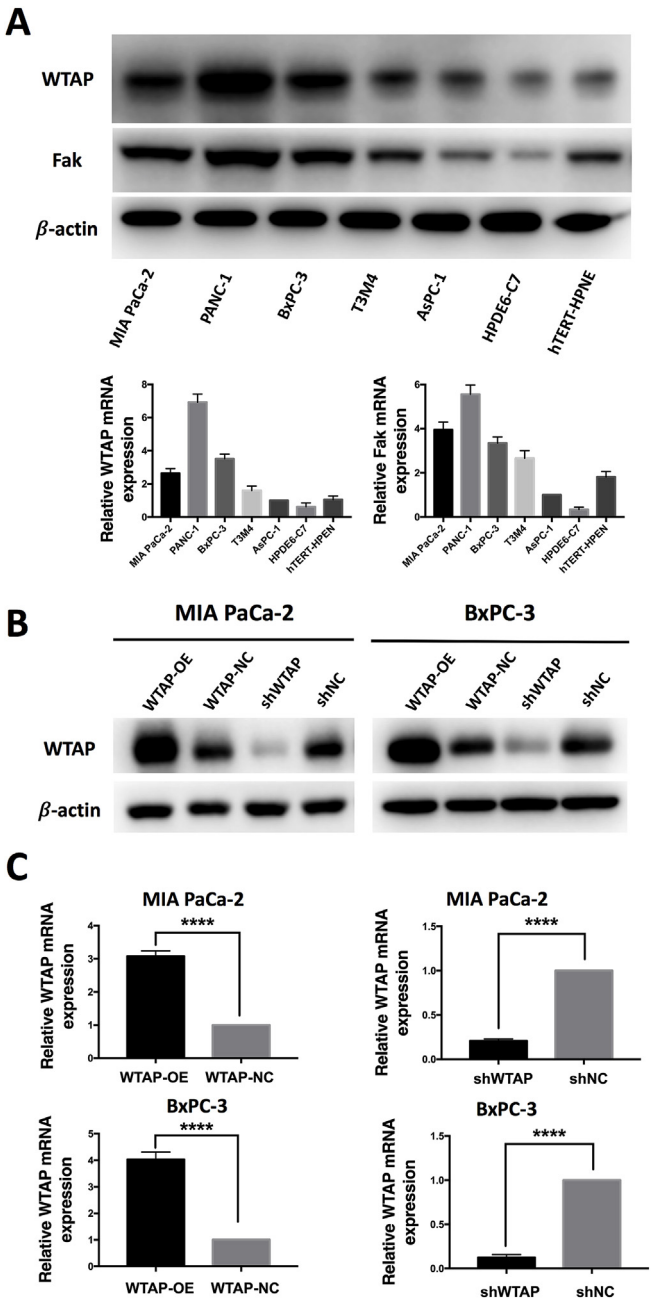
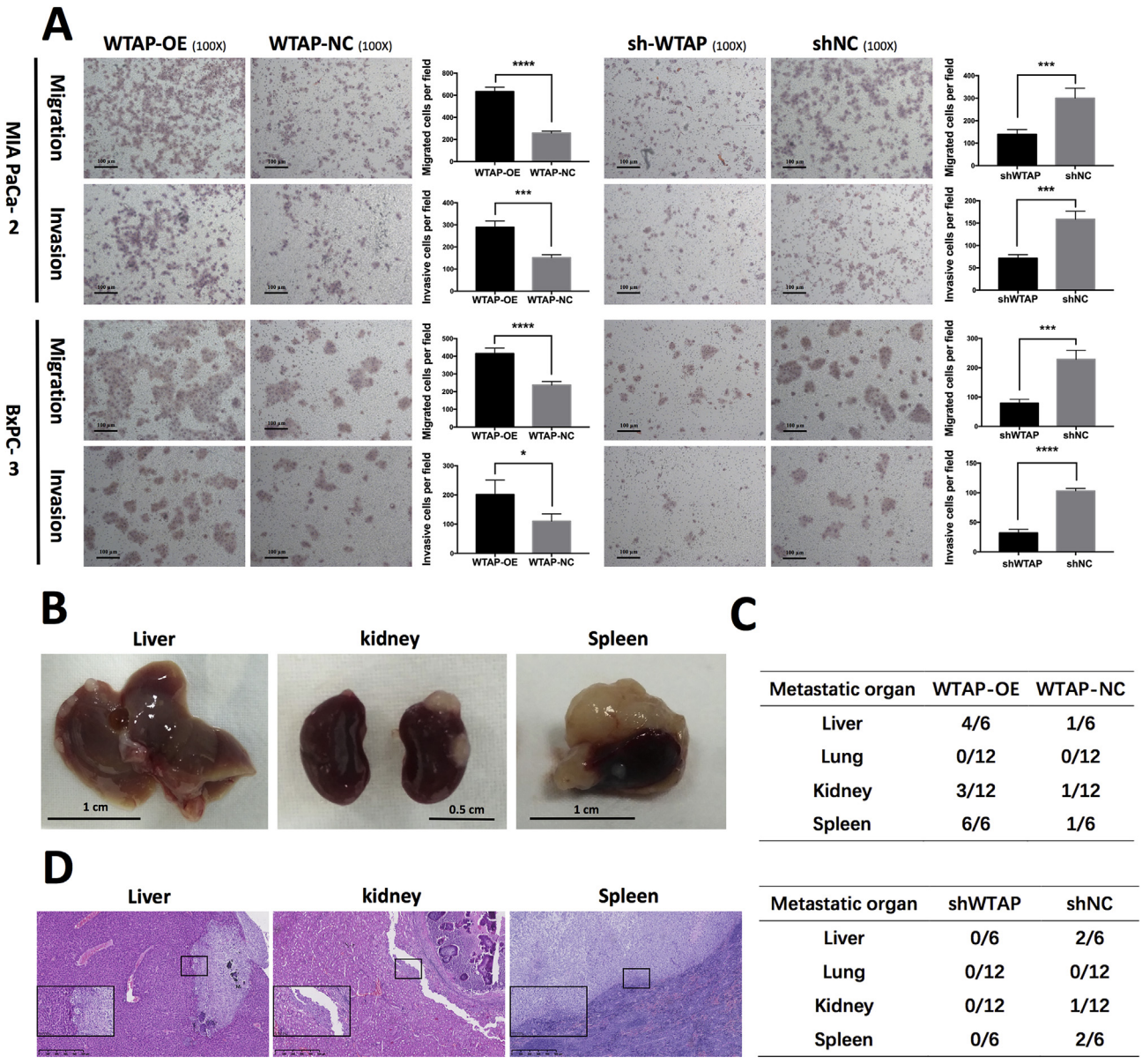


Fig. 1. MIA PaCa-2 and BxPC-3 were selected as tool cells; mono-clonal stable transductions of both WTAP overexpression and knockdown were established. (A) WTAP/Fak mRNA and protein expression in five PC cells lines and two normal pancreatic duct epithelial cell lines. (B, C) The efficiency of WTAP overexpression and WTAP knockdown were confirmed by western blot and qRT-PCR. The data are presented as the mean ± SD. (Student's t-test; ****, $p < 0.0001$) WTAP: WT1 associated protein; Fak: focal adhesion kinase; WTAP-OE: WTAP overexpression; WTAP-NC: WTAP negative control; shWTAP: WTAP knockdown; shNC: scramble control.



Metastatic organ	WTAP-OE	WTAP-NC
Liver	4/6	1/6
Lung	0/12	0/12
Kidney	3/12	1/12
Spleen	6/6	1/6

Metastatic organ	shWTAP	shNC
Liver	0/6	2/6
Lung	0/12	0/12
Kidney	0/12	1/12
Spleen	0/6	2/6

Fig. 2. WTAP promoted PC cell migration and invasion both in vitro and in vivo. (A) PC cell migration/invasion was assessed by transwell assay (magnification: 100 ×). (B) Liver, kidney and spleen metastasis obtained from orthotopic xenograft mouse model. (C) The numbers of metastatic lesions. (D) H&E staining was performed to validate the liver, kidney and spleen metastasis pathologically (magnification: 50 × /200 ×). The data are presented as the mean ± SD. (Student's t-test; *, $p < 0.05$; ***, $p < 0.001$ and ****, $p < 0.0001$) WTAP: WT1 associated protein; PC: pancreatic cancer; H&E: hematoxylin and eosin; WTAP-OE: WTAP overexpression; WTAP-NC: WTAP negative control; shWTAP: WTAP knockdown; shNC: scramble control.

respectively using MIA PaCa-2 and BxPC-3 cell lines. The efficiency of WTAP-overexpression and WTAP-knockdown compared to the corresponding control cell lines was further confirmed by western blot (Fig. 1B) and qRT-PCR (Fig. 1C).

3.2. WTAP promoted PC cell migration and invasion both in vitro and in vivo

In vitro transwell assay was employed to evaluate the impact of WTAP on PC cell motility. As shown in Fig. 2A, cell migration and invasion was increased by WTAP overexpression and suppressed by WTAP knockdown in both MIA PaCa-2 and BxPC-3 cells. To further examine the effects of WTAP on tumor metastasis in vivo, we established orthotopic xenograft mouse model. Eight weeks later, the animals were sacrificed, WTAP expression was examined by IHC (Supplementary Fig. 1), and liver, lung, kidney and splenic metastasis were obtained (Fig. 2B). As expected, the metastatic capacity of WTAP-OE MIA PaCa-2 cells was elevated when compared with that of WTAP-NC MIA PaCa-2 cells (Fig. 2C). Consistently, the metastatic capacity of shWTAP MIA PaCa-2 cells was suppressed when compared with that of shNC MIA PaCa-2 cells (Fig. 2C). Further H&E staining was performed to validate the hepatic, renal and splenic metastasis pathologically (Fig. 2D). In combination, these findings indicated that WTAP promoted the metastatic capacity of PC cells both in vitro and in vivo.

As previous study showed that WTAP could promote cell proliferation in RCC [16], we also investigated its role on cell proliferation in PC. The cell proliferation assay and cell cycle assay were conducted, however, no difference were observed when comparing WTAP-OE/shWTAP group with the control groups (Supplementary Figs. 2A–B). In addition, no difference was observed on the weight/volume of primary tumors (Supplementary Figs. 2C–E).

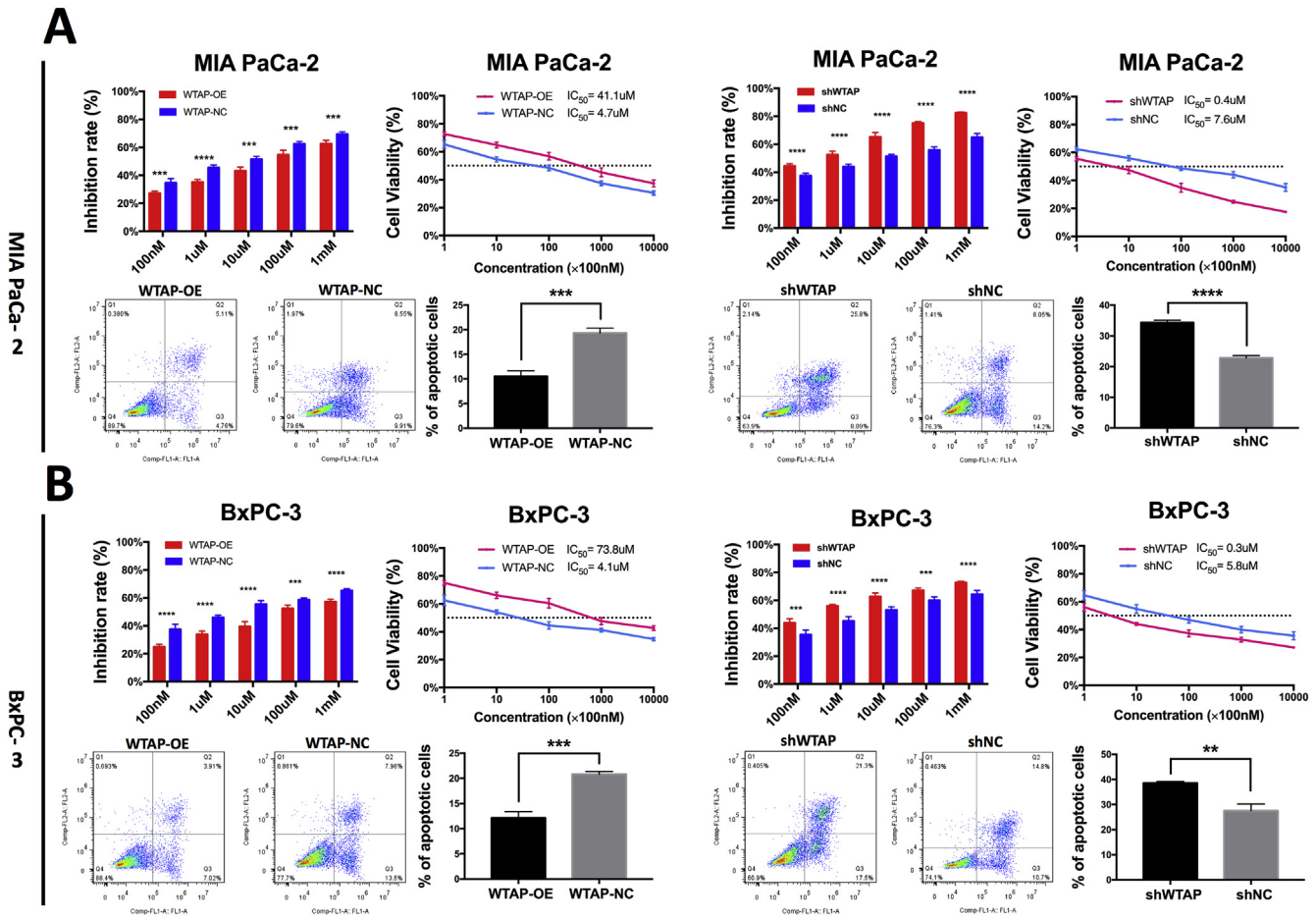


Fig. 3. WTAP increased chemo-resistance to gemcitabine in vitro. (A) WTAP over-expression decreased chemo-sensitivity to gemcitabine and WTAP knockdown increased chemo-sensitivity to gemcitabine in MIA PaCa-2 cells. (B) WTAP over-expression decreased chemo-sensitivity to gemcitabine and WTAP knockdown increased chemo-sensitivity to gemcitabine in BxPC-3 cells. The data are presented as the mean ± SD. (Student's t-test; **, P < 0.01; ***, p < 0.001 and ****, p < 0.0001) WTAP: WT1 associated protein; WTAP-OE: WTAP overexpression; WTAP-NC: WTAP negative control; shWTAP: WTAP knockdown; shNC: scramble control; IC50: the half maximal inhibitory concentration.

3.3. WTAP increased chemo-resistance to gemcitabine in vitro

Cytotoxicity assay was carried out to investigate the impact of WTAP on gemcitabine chemo-sensitivity in PC cells. As shown in Fig. 3A, WTAP overexpression decreased the gemcitabine inhibition rate in MIA PaCa-2 cells. The half maximal inhibitory concentration (IC50) of gemcitabine in WTAP-OE group (41.1 μM) was higher than that in the control group (4.7 μM). Conversely, WTAP knockdown increased the gemcitabine inhibition rate in MIA PaCa-2 cells. The IC50 of gemcitabine in shWTAP group (0.4 μM) was lower than that in the shNC group (7.4 μM). Further cell apoptosis assay confirmed the above results. After treated with gemcitabine (10 μM) for 48 h, MIA PaCa-2 cells infected by WTAP-OE lentivirus had a lower apoptosis rate than the control cell lines, whereas the percentage of apoptosis of shWTAP lentivirus infected MIA PaCa-2 cells was significantly higher than that of shNC lentivirus infected MIA PaCa-2 cells (Fig. 3A). In addition, similar results were also observed in BxPC-3 cells (Fig. 3B). In summary, these data indicated that WTAP promoted PC cells' chemo-resistance to gemcitabine in vitro.

3.4. WTAP regulated Fak expression and activated Fak signaling pathway in PC cells

To investigate how WTAP regulated the cell mobility and chemo-resistance to gemcitabine of PC cells, qRT-PCR was performed to detect the Fak mRNA. We found that the Fak mRNA of WTAP-OE group was significantly higher that of the control group in both MIA PaCa-2 and BxPC-3 cells (Fig. 4A). In contrast, WTAP knockdown had an opposite effect on Fak mRNA (Fig. 4A). We then measured the Fak expression using western blot, which demonstrated that Fak expression was positively correlated with WTAP expression (Fig. 4B). Next, we found the expression of WTAP and Fak was positively correlated in PC tissues by using GEPIA (N of PC patients = 179, 2-tailed Spearman's correction, R = 0.2, P = 0.0086) (Supplementary Fig. 3).

Furthermore, we also examined the protein levels of key components, which are involved in the regulation of cell movement and chemo-sensitivity, within the Fak pathway. Western blot revealed that the phosphorylated form of Fak, Src, AKT and Erk1/2 were increased in WTAP-OE PC cells and decreased in shWTAP PC cells (Fig. 4B). Thus, all these data indicated that WTAP increased Fak expression and, as a result, activated Fak signaling pathway.

3.5. WTAP stabilized Fak mRNA by directly binding to Fak mRNA

To determine how WTAP increased Fak mRNA, whether through enhancing Fak mRNA stability or through promoting Fak gene transcription, we performed Fak mRNA stability assay using actinomycin D (10μg/mL). As Fig. 5A illustrated, after actinomycin D treatment, the percentage of remaining Fak mRNA was higher in WTAP-OE group and lower in shWTAP group when compared with the control group respectively. These phenomena were observed in both MIA PaCa-2 and BxPC-3 cells, which suggested that WTAP could increase the stability of

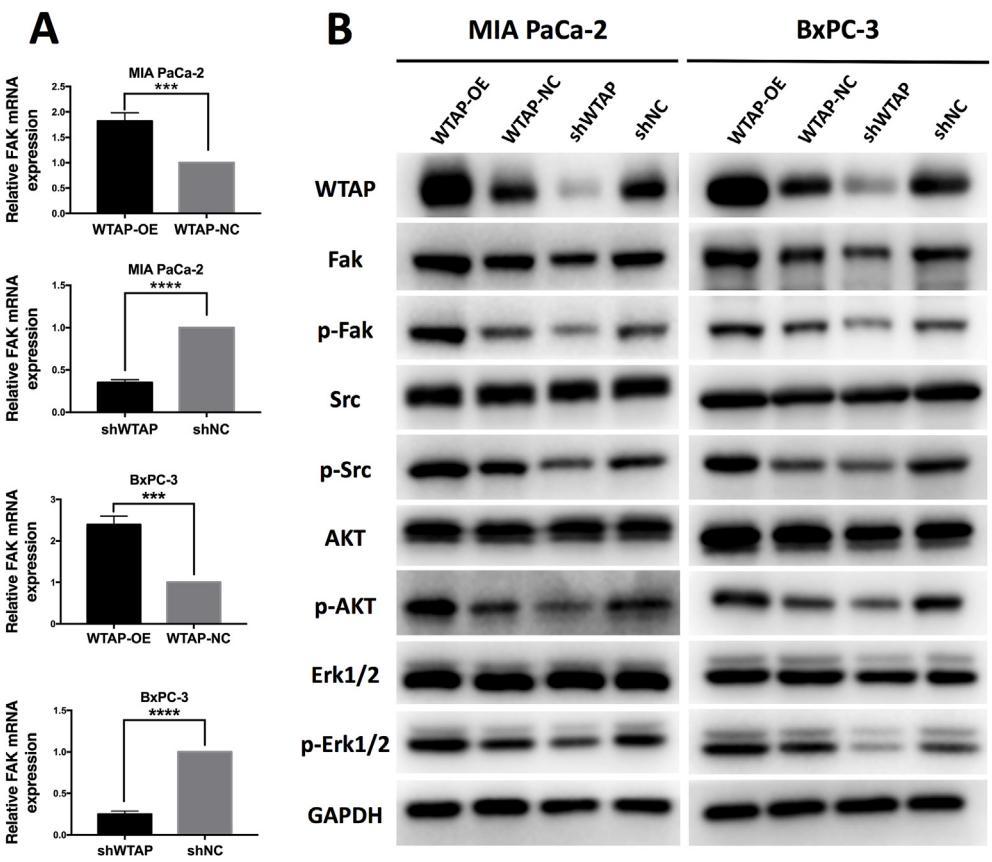


Fig. 4. WTAP regulated Fak expression and activated Fak signaling pathway in PC cells. (A) WTAP overexpression increased Fak mRNA in MIA PaCa-2 and BxPC-3 cells, while WTAP knockdown had an opposite effect. (B) The key components in Fak signaling pathway were evaluated by western blot, and the data indicated that WTAP increased Fak expression and activated Fak signaling pathway. The data are presented as the mean ± SD. (Student's t-test; ***, $p < 0.001$ and ****, $p < 0.0001$) WTAP: WT1 associated protein; Fak: focal adhesion kinase; WTAP-OE: WTAP overexpression; WTAP-NC: WTAP negative control; shWTAP: WTAP knockdown; shNC: scramble control.

Fak mRNA.

Further RIP was performed to explore whether WTAP stabilized Fak mRNA by directly binding to it. MIA PaCa-2 and BxPC-3 cells were lysed and immunoprecipitated with protein A/G magnetic beads conjugated to anti-WTAP anti-body or normal rabbit IgG at 4 °C overnight. After RNA purification, qRT-PCR analysis demonstrated that Fak mRNA was significantly enriched in the WTAP immunocomplexes, but not in the normal rabbit IgG immunocomplexes (Fig. 5B). These findings indicated that WTAP could bind to Fak mRNA and thereby enhanced its stability (Fig. 5C).

3.6. GSK2256098 reversed the WTAP-induced cell migration and invasion in PC cells

The WTAP-OE cells were cultured in medium containing various amount of GSK2256098 (0–10 μM), while the WTAP-NC cells were cultured in the standard medium for 48 h. Aiming to select an appropriate GSK2256098 concentration for the following inhibitor experiment, the Fak and p-Fak were detected by western blot after GSK2256098 treatment. Finally, we chose 5 μM for WTAP-OE MIA PaCa-2 cells and 2 μM for WTAP-OE BxPC-3 cells (Fig. 6A). Further western blot assay showed that the p-Fak, p-Src, p-AKT and p-Erk1/2 of WTAP-OE group were decreased and lower than those of WTAP-NC group after GSK2256098 treatment (Fig. 6B), which demonstrated that GSK2256098 could reverse WTAP-induced Fak signaling pathway activation.

To identify the effect of Fak signaling pathway on cell mobility in PC cells, we then repeated the transwell assays in WTAP-OE cells treated with GSK2256098 and WTAP-NC cells. We found that after GSK2256098 treatment, the number of migrated/invasive cells of WTAP-OE group was smaller than that of control group (Fig. 6C). These results indicated that the Fak signaling pathway mediated WTAP-induced cell migration and invasion, which could be reversed by Fak inhibitor.

3.7. GSK2256098 reversed the WTAP-induced chemo-resistance to gemcitabine in PC cells

To confirm the contribution of Fak signaling pathway in WTAP-induced chemo-resistance to gemcitabine, cytotoxicity assay and cell apoptosis assay were conducted in WTAP-OE cells treated with GSK2256098, WTAP-OE cells and WTAP-NC cells. We found that the gemcitabine inhibition rate of WTAP-OE group, treated with GSK2256098, was higher than that of WTAP-NC group (Fig. 6D). Consistently, the IC50 of gemcitabine in WTAP-OE group, treated with GSK2256098, was lower than that in WTAP-OE and WTAP-NC group. Further cell apoptosis assay, which revealed consistent result with cytotoxicity assay, showed that the percentage of apoptotic cells in WTAP-OE group, treated with GSK2256098, was higher than that of WTAP-OE and WTAP-NC group (Fig. 6D). The above results proved that WTAP-induced chemo-resistance to gemcitabine in PC cells was, at least partially, driven by Fak signaling pathway and could be blocked by GSK2256098.

4. Discussion

In our previous investigation, we found that WTAP was a novel prognostic factor in PC [17]. For one thing, the nuclear WTAP expression in tumor tissues was significantly higher than that of non-tumor tissues. For another, patients with high WTAP expression had a dismal prognosis when compared with patients who had low WTAP expression. In our present study, we demonstrated that WTAP could enhance PC cell migration/invasion and suppress its chemo-sensitivity to gemcitabine in a Fak-dependent manner. In detail, WTAP could bind to Fak mRNA and enhance its stability. Subsequent Fak overexpression then induced the activation of Fak signaling pathway, including Fak-

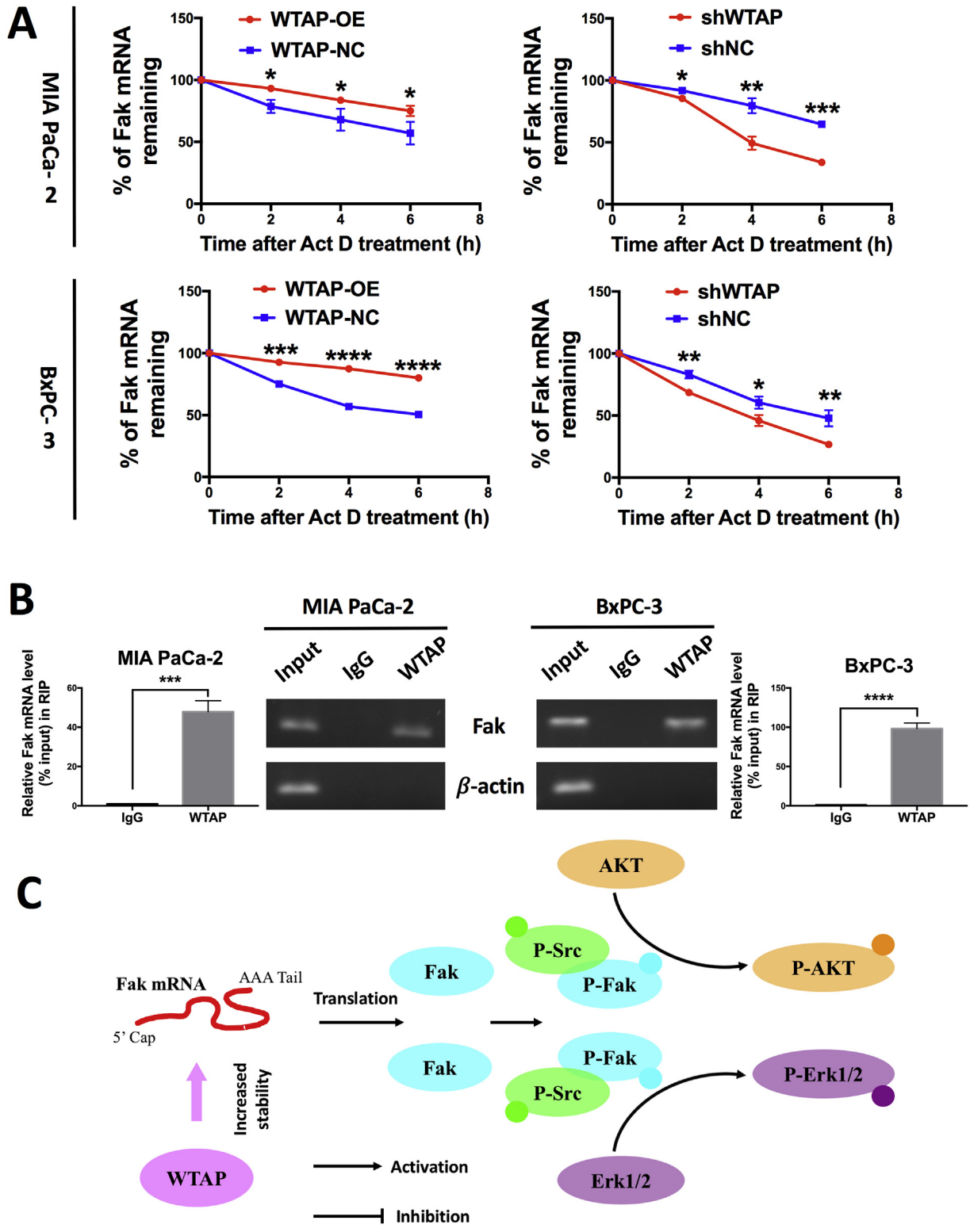


Fig. 5. WTAP stabilized Fak mRNA by directly binding to Fak mRNA. (A) The stability of Fak mRNA was determined by qRT-PCR after actinomycin D (10μg/mL) treatment. (B) PC cell lysates were immunoprecipitated with WTAP antibody or control IgG followed by RT-PCR and qRT-PCR. (C) Schematic representation of WTAP-induced signaling molecules involved in regulating PC cell metastasis and chemo-resistance. The data are presented as the mean ± SD. (Student's t-test; *, $p < 0.05$, **, $p < 0.01$, ***, $p < 0.001$ and ****, $p < 0.0001$) WTAP: WT1 associated protein; Fak: focal adhesion kinase; PC: pancreatic cancer; Act D: actinomycin D; WTAP-OE: WTAP overexpression; WTAP-NC: WTAP negative control; shWTAP: WTAP knockdown; shNC: scramble control.

PI3K-AKT and Fak-Src-GRB2-Erk1/2 pathways. GSK2256098, a small molecular Fak inhibitor, could reverse WTAP-induced tumor cell migration/invasion and chemo-resistance to gemcitabine in PC by suppressing the phosphorylation of AKT and Erk1/2. As such, we suggest that WTAP plays its carcinogenic role in PC by activating Fak signaling pathway and Fak inhibition might be a promising therapy for PC patients with WTAP over-expression.

Since WTAP was first identified as a prognostic factor in

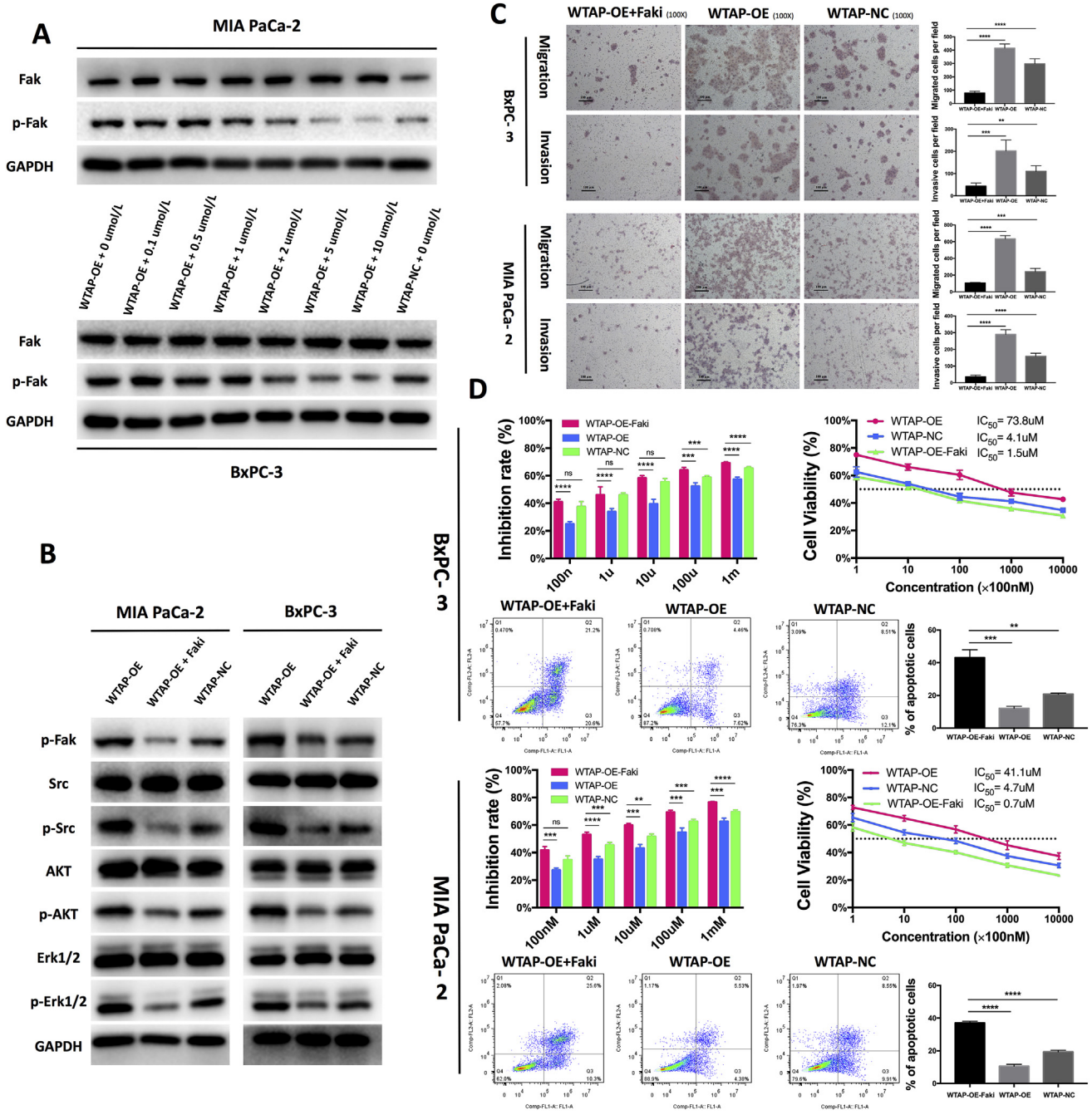


Fig. 6. GSK2256098 reversed WTAP-induced cell migration/invasion and chemo-resistance to gemcitabine in PC cells. (A) Appropriate GSK2256098 concentration was selected according to the western blot results. (B) The key components in Fak signaling pathway were evaluated by western blot after GSK2256098 treatment. (C) The PC cell migration and invasion were measured by transwell assay after GSK2256098 treatment (magnification: 100 ×). (D) Cytotoxicity assay and cell apoptosis assay proved that WTAP-induced chemo-resistance to gemcitabine in PC cells could be blocked by GSK2256098. The data are presented as the mean ± SD. (Student's t-test; **, $p < 0.01$; ***, $p < 0.001$ and ****, $p < 0.0001$) WTAP: WT1 associated protein; Fak: focal adhesion kinase; PC: pancreatic cancer; Faki: Fak inhibitor (GSK2256098); WTAP-OE: WTAP overexpression; WTAP-NC: WTAP negative control; IC50: the half maximal inhibitory concentration; ns: no significance.

glioblastoma [12], an increasing number of studies focused on its oncogenic role in various types of malignant tumors. For example, high WTAP expression was associated with advanced TNM staging in cholangiocarcinoma which was in accordance with in vitro assay result that WTAP could promote cholangiocarcinoma cells migration and invasion [13]. Further cDNA microarray analysis indicated that MMP7, MMP28, Cathepsin H and Muc1might be its downstream targets. In CRC, WTAP was overexpressed as a result of epigenetic inactivation of the carbonic anhydrase IV gene [15]. The transcription of TBL1, a target gene of WT1 [28], decreased as the overexpressed WTAP antagonized the transcriptional activity of WT1, which led to the activation of Wnt/β-catenin signaling pathway. Abnormal activation of Wnt/β-catenin signaling pathway then mediated the proliferation, invasion and migration of colon cancer cells. Moreover, WTAP was also a prognostic indicator for RCC patients and played its oncogenic role in RCC by binding to and stabilizing CDK2 transcript [16]. Besides solid tumors, the oncogenic role of WTAP was also observed in AML [14]. The mTOR pathway, identified as another WTAP downstream target, mediated its important impact on proliferation, survival and differentiation blockage in AML cells. Our laboratory previously found that WTAP was an independent

prognostic factor in PC and its high expression usually predicted a poor prognosis in PC patients [17]. This study further demonstrated that WTAP could promote PC cell migration and invasion both in vitro and in vivo. In addition, we also found that WTAP decreased chemo-sensitivity to gemcitabine in vitro. However, the underlying molecular mechanism is still unknown.

Besides its carcinogenic role, WTAP was also discovered to participate in several physical processes in normal cells. As previously reported, WTAP was involved in cell proliferation, survival, apoptosis [7,29,30], eye development [8] and embryonic development [7] owing to its complex function in transcriptional and post-transcriptional regulation. Firstly, as a WT1 associated protein, WTAP could bind to WT1 to inhibit its transcription factor activity which in turn suppressed TBL1 [15], amphiregulin and Bcl-2 [29] transcription. Secondly, as an important component of spliceosome [31], WTAP was reported to regulate the alternative splicing of survivin pre-mRNA in vascular smooth muscle cells [30]. Thirdly, WTAP could enhance the stability of cyclin A2 and CDK2 mRNA stability by binding to their 3′-UTR [7,16]. However, on the contrary, WTAP might also accelerate the degradation of some of its target mRNAs by promoting m6A formation [32,33]. Fak, a non-receptor protein tyrosine kinase, can be dimerized and activated by overexpression [19]. Several studies have confirmed its pro-metastatic role and the increased chemo-resistance to gemcitabine induced by Fak in PC [22–27]. Moreover, Fak expression can be subject to transcriptional regulation [34,35], alternative splicing [36,37], and mRNA stability [38]. Our preliminary data showed that WTAP overexpression could increase Fak and Fak mRNA in PC cells, while WTAP knockdown revealed an opposite effect. Taking together, we presumed that Fak was very likely to be the downstream target of WTAP in PC. In order to determine whether WTAP regulate Fak expression through a transcriptional or post-transcriptional manner, we conducted the Fak mRNA stability assay using actinomycin D. And the results indicated that Fak mRNA could be stabilized by WTAP. Further RIP assay demonstrated that WTAP could bind to WTAP mRNA specifically. Previous studies found WTAP could stabilize cyclin A2 and CDK2 mRNA by binding to their 3′-UTR [7,16]. And it has been proved that ACAAAU UAU, which corresponds to the 3′-UTR 1526–1534 in cyclin A2 mRNA, was the specific binding sequence for WTAP [7]. Then the sequence of Fak mRNA was obtained from NCBI (https://www.ncbi.nlm.nih.gov) (Supplementary file. 2) and a similar sequence, ACAAAGAAU (3′-UTR 4025–4033) was identified. It is very likely that ACAAAGAAU (3′-UTR 4025–4033) is an essential element required for the WTAP-mediated stabilization of Fak mRNA which needs to be confirmed by further study. Next, with the concern that whether WTAP-induced Fak overexpression could result in the activation of itself and its downstream signaling pathways, western blot was performed. Not surprisingly, the western blot results confirmed that the WTAP could activate both Fak-PI3K-AKT and Fak-Src-GRB2-Erk1/2 signaling pathways.

GSK2256098 has been demonstrated to effectively inhibit Fak-PI3K-AKT and Fak-Src-GRB2-Erk1/2 signaling pathways in PC [39]. Our study revealed a similar result using western blot. In addition, we also found that after GSK2256098 treatment, the chemo-resistance to gemcitabine and pro-metastatic effect induced by WTAP overexpression could be completely reversed. Thus, we presumed that Fak inhibitor might be a promising option for PC patients with WTAP overexpression.

In summary, we demonstrated that WTAP could promote migration/invasion and chemo-resistance to gemcitabine in PC by stabilizing Fak mRNA and activating Fak signaling pathway. Moreover, small molecular Fak inhibitor could reverse its oncogenic role in PC. Therefore, Fak signaling pathway might be a promising therapeutic target in PC patients. However, the specific binding sequence for WTAP in Fak mRNA should be investigated and the in vivo anti-tumor efficacy of GSK2256098 should be confirmed in the further study.

Authors' contributions

BQL, JCG and YPZ managed the experimental design; BQL and JCG carried out the experiments and drafted the manuscript; ZYL contributed to the pathological diagnosis and immunohistochemistry experiments; LY was involved in the statistical analysis; QFL, SS, TPZ and YPZ reviewed the manuscript and made critical revision for important intellectual content; JCG and YPZ provided funding support. All authors read and approved the final version of the manuscript.

Conflicts of interest

The authors have no conflict of interest.

Acknowledgements

The authors acknowledge the supporting from Dr. Bo-Ju Pan, Dr. Cheng-Cheng Wang and Dr. Jun-Ze Pang. This study was supported by grants from the CAMS Innovation Fund for Medical Sciences (CIFMS, 2016-I2M-3-019) and Non-profit Central Research Institute Fund of Chinese Academy of Medical Sciences (No. 2018PT32014).

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.canlet.2019.02.043.

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