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Aberrant expression of PDZ-binding kinase modulates human pancreatic cancer cell invasion

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Abstract

Pancreatic ductal adenocarcinoma (PDAC) is the fourth leading cause of cancer-related deaths and is projected to be the second leading cause of cancer deaths by 2030. Incidence to mortality ratio for PDAC is almost one, suggesting its aggressive phenotype. The poor survival rate of PDAC patients is due to metastasis to distant organs and, therefore, recent focus has been aimed towards the identification of molecular targets that drive metastatic disease. We are investigating an under-studied kinase, PDZ-domain binding kinase (PBK), as a target for pancreatic cancer (PanCa) therapy. PBK is a dual-specificity serine/threonine kinase involved in mitosis and invasive behavior of cancer cells[1]. It is expressed physiologically in the testis, but aberrant expression results in an aggressive phenotype in a variety of cancers[2,3]. Our results demonstrate that PBK expression varies across a panel of human PanCa cell lines, and directly correlates with their invasive ability. Similarly, a panel of patient-derived primary PanCa shows higher PBK levels associated with aggressive behavior and invasion. To assure specificity of PBK to this invasive phenotype, we measured the effect of PBK overexpression or knockdown on the PanCa lines' invasive ability. Overexpression of PBK in low PBK expressing cells increased PanCa invasion ~10-fold, while PBK knockdown reduced invasive ability ~5-fold. Immunohistochemical analyses of resected human PanCa specimens revealed that PBK is present in the majority of human PanCa tissue samples tested, but not in the adjacent normal pancreatic tissue. Interestingly, aberrant PBK localization was found in even early stages of PanCa development and was sustained throughout its progression. In search of molecular effectors of invasion regulated by PBK, we examined various genes/proteins that are involved in cancer cell invasion and metastasis. RelB, a well-known metastatic inducer in various cancers[4,5], was found to be a major player in PanCa and regulated by PBK. These data indicate a novel molecular pathway of PBK, where its presence plays a role in the invasion of human PanCa cells. Therefore, the role of PBK in promoting cancer cell invasion, combined with its general lack of expression in normal cells, nominates PBK as a potentially important therapeutic target for pancreatic cancer.

Methods

OTS-514: A novel pharmacological inhibitor of PBK, OTS-514, was used to modulate PBK kinase activity in a variety of established and patient derived pancreatic cancer cell lines. OTS-514 is a thieno[2,3-c]quinolone compound and has been proven to be a highly potent PBK inhibitor[2].

Cell Culture: Human pancreatic cancer cell lines were cultured in phenol red-free IMEM, penicillin-streptomycin and 10% FBS. PDX cells were cultured in Advanced MEM, glutamine, penicillin-streptomycin and 10% FBS. All cells were treated with 100nM of OTS-514 for 72 hours for further analyses.

Immunoblots: Cell lysates were prepared using RIPA buffer and protein concentrations measured by Bradford assay. Fifty micrograms of proteins were resolved 10% SDS-PAGE gels, transferred to nitrocellulose membranes, blocked and treated with primary and secondary antibodies. Membranes were visualized in a Fujifilm LAS-1000 imager using ECL substrate.

Expression vectors and siRNA: PBK expression vector was purchased from GenTarget Inc., transformed into *E.coli* and plasmid DNA was purified using a Qiagen Endotoxin-free plasmid purification kit. Concentration and purity was measured using NanoDrop. PANC-1 cells were transfected using GenJet, according to manufacturers protocol. Expression of PBK was verified by immunoblot. On-Target SMARTpool siRNA's against PBK and RelB were purchased from Dharmacon and PANC-1 cells were transfected using PepMute transfection reagent and cultured for 72 hours.

Gelatin zymography: Cells were cultured in 100mm plates to ~70% confluence, media was changed to serum-free media and culture was continued for an additional 16-18 hours. Cells were counted and cultured media was collected. 1ml of media per sample was concentrated using centrifugal filters and combined with sample buffer. 20µl's of concentrated media were loaded into gelatin zymogram gels, resolved using electrophoresis, treated with renaturation solution for 30min, washed, treated in developing buffer for 30min then incubated overnight at 37°C in fresh developing buffer. Gels were then stained with 0.4% Coomassie blue, visualized and photographed.

Immunohistochemistry: Archival tissue sections from Histopathology & Tissue Shared Resource, Lombardi Cancer Center, Georgetown University Medical center, were treated with 1:250 dilution of various antibodies at 4°C overnight, followed by incubation with 1:3,000 dilution secondary antibody for 1hr. Tissue sections were then incubated in ABC-P Reagent for 30 min. followed by treatment with 3,3'-diaminobenzidine (DAB). Tissue sections were counterstained with hematoxylin, mounted and photographed.

Cell Invasion Assays: Performed using Corning Matrigel Invasion Chamber, according to manufacturer's protocol.

XTT Assay: Cell viability/growth was measured by an XTT assay kit purchased from Sigma and performed in triplicate according to manufacturer's protocol.

Quantification and statistical analysis: Pixel densities of Western blots and Gelatin zymograms were measured using ImageJ and statistical analysis was performed in GraphPad Prism 7. Student's t-test was used and $p < 0.05$ were considered significant.

Results

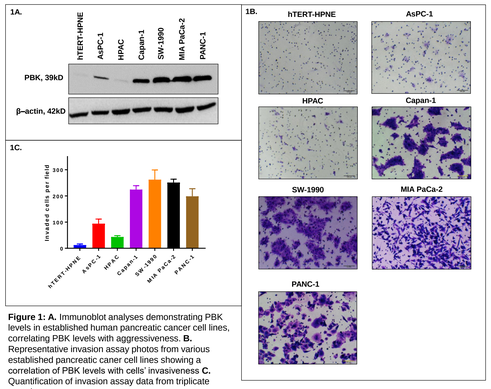

Figure 1: A. Immunoblot analyses demonstrating PBK levels in established human pancreatic cancer cell lines, correlating PBK levels with aggressiveness. **B.** Representative invasion assay photos from various established pancreatic caner cell lines showing a correlation of PBK levels with cells' invasiveness **C.** Quantification of invasion assay data from triplicate experiments.

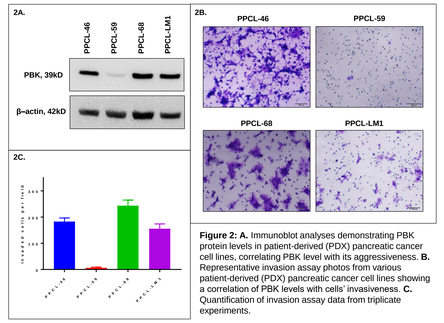

Figure 2: A. Immunoblot analyses demonstrating PBK protein levels in patient-derived (PDX) pancreatic cancer cell lines, correlating PBK level with its aggressiveness. **B.** Representative invasion assay photos from various patient-derived (PDX) pancreatic cancer cell lines showing a correlation of PBK levels with cells' invasiveness. **C.** Quantification of invasion assay data from triplicate experiments.

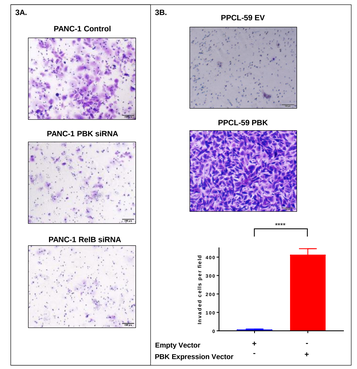

Figure 3: A. Representative photos showing invasiveness of PANC-1 cells with or without PBK or RelB siRNA transfection. **B.** Invasion assay evaluating effect of PBK over-expression on cell invasiveness, comparing PPCL-59 empty vector cells (low endogenous levels of PBK) to PPCL-59 PBK transfected cells. Representative photos from triplicate experiments. Quantification data showing significant difference, $p < 0.0001$.

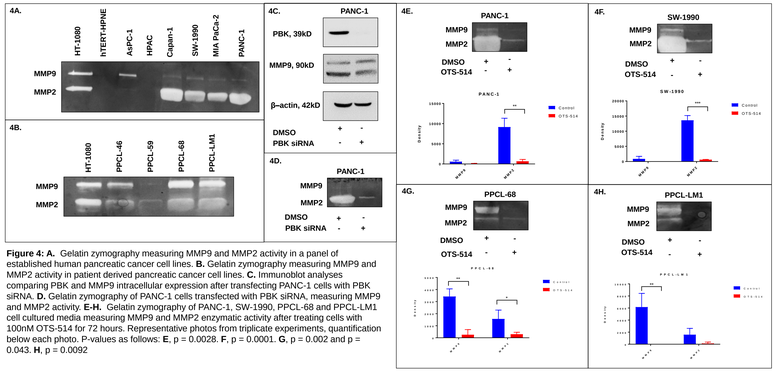

Figure 4: A. Gelatin zymography measuring MMP9 and MMP2 activity in a panel of established human pancreatic cancer cell lines. **B.** Gelatin zymography measuring MMP9 and MMP2 activity in patient derived pancreatic cancer cell lines. **C.** Immunoblot analyses comparing PBK and MMP9 intracellular expression after transfecting PANC-1 cells with PBK siRNA. **D.** Gelatin zymography of PANC-1 cells transfected with PBK siRNA, measuring MMP9 and MMP2 activity. **E-H.** Gelatin zymography of PANC-1, SW-1990, PPCL-68 and PPCL-LM1 cell cultured media measuring MMP9 and MMP2 enzymatic activity after treating cells with 100nM OTS-514 for 72 hours. Representative photos from triplicate experiments, quantification below each photo. P-values as follows: **E**, $p = 0.0028$. **F**, $p = 0.0001$. **G**, $p = 0.002$ and $p = 0.043$. **H**, $p = 0.0092$

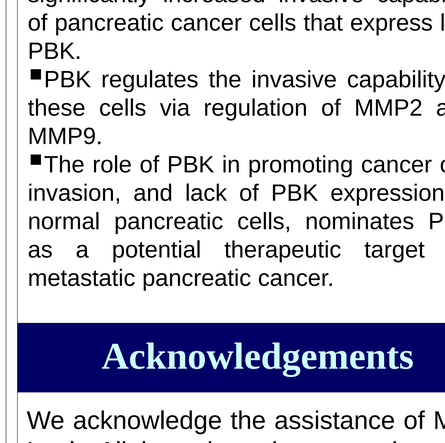

Figure 5: A. Immunoblot analyses evaluating endogenous RelB levels in established human pancreatic cell lines. **B.** Immunoblot analyses evaluating endogenous RelB levels in patient derived pancreatic cancer cell lines. **C.** Immunoblot analyses demonstrating PBK knockdown resulting in down-regulation of RelB, using PBK siRNA. **D.** Immunoblot analyses demonstrating RelB knockdown resulting in down-regulation of intracellular MMP9, using RelB siRNA. **E.** Gelatin zymography demonstrating diminished MMP9 and MMP2 enzymatic activity in cultured media from PANC-1 cells transfected with RelB siRNA.

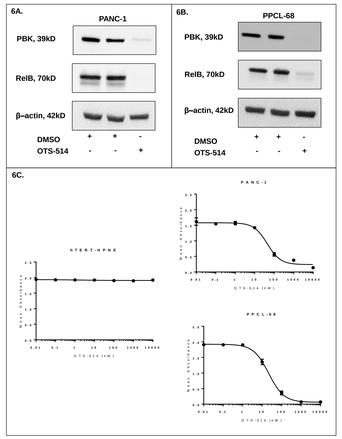

Figure 6: A. Immunoblot analyses of PANC-1 cells treated with 100nM OTS-514 for 72 hours, demonstrating down-regulation of both PBK and RelB. **B.** Immunoblot analyses of PPCL-68 PDX cells treated with 100nM OTS-514 for 72 hours, demonstrating down-regulation of both PBK and RelB. **C.** IC-50 curves for hTERT-HPNE, PANC-1 and PPCL-68 cells, demonstrating lack of growth inhibitory effect in non-tumorigenic cells and efficacy in cell lines that express PBK.

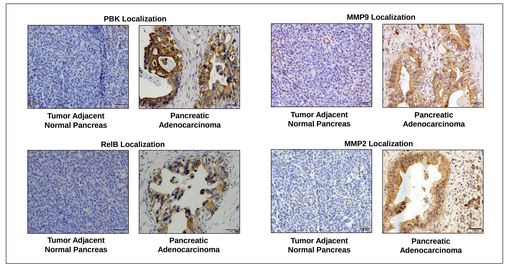

Figure 7: Immunohistochemical staining of tumor adjacent normal pancreas and human pancreatic carcinoma tissue for PBK, RelB, MMP9 and MMP2. PBK, RelB, MMP9 and MMP2 expression is increased in pancreatic carcinoma as compared to tumor adjacent normal pancreatic tissue.

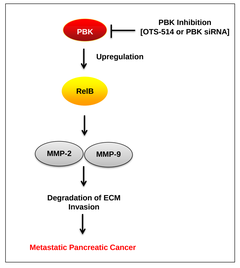

Figure 8: Cartoon showing proposed signaling pathway by which PBK regulates MMPs and invasion/metastasis. PBK up-regulates RelB which in turn regulates MMP9 and MMP2, leading to degradation of extracellular matrix and thereby invasion and metastasis of pancreatic cancer cells.

Conclusions

- Our data indicate, for the first time, that increased PBK protein levels in pancreatic carcinoma is correlated with the with the invasive capability of PDAC cells.
- This association exists in both established human pancreatic cell lines and patient derived pancreatic cancer cells.
- PBK inhibition results in diminished invasive capability of treated cells (both pharmacologically and genetically) while PBK over-expression results in significantly increased invasive capability of pancreatic cancer cells that express low PBK.
- PBK regulates the invasive capability of these cells via regulation of MMP2 and MMP9.
- The role of PBK in promoting cancer cell invasion, and lack of PBK expression in normal pancreatic cells, nominates PBK as a potential therapeutic target for metastatic pancreatic cancer.

Acknowledgements

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