P21 activated kinase 2 promotes pancreatic cancer growth and metastasis

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Abstract. Pancreatic cancer has an overall 5-year survival rate of only 9%, due to its rapid metastasis and poor prognosis. To combat this disease, novel therapeutic targets and biomarkers are required. In this study, immunohistochemistry was used to detect the expression of P21 activated kinase 2 (PAK2) protein in the tissues of cancer and the paired adjacent normal tissues. The association between PAK2 and the clinicopathologic features of patients with pancreatic cancer was subsequently analyzed. The results indicated that PAK2 was overexpressed in the cancer tissues, which indicated high pTNM stage, poor tumor grade, lymph node metastasis and vascular invasion. In addition, the results demonstrated evidence of a close association between PAK2 expression and poor prognosis of patients with pancreatic cancer. The results also suggested that PAK2 may promote pancreatic cancer cell proliferation and migration in vitro through clone formation, MTT, wound healing and Transwell assays. The present study further identified that PAK2 could stimulate pancreatic cancer growth and metastasis in mice. Decreased expression of proliferation marker protein Ki-67 and proliferating cell nuclear antigen in response to PAK2 knockdown further verified the role of PAK2 in promoting cell proliferation by western blot analysis. In addition, the expression levels of matrix metallopeptidase (MMP) 2 and MMP9 were decreased in PANC1 and BxPC3 cell lines transfected with PAK2-short hairpin RNA as indicated in western blot analysis, suggesting a function of PAK2 in promoting cell invasion. Collectively, these findings revealed a critical role for PAK2 in the development of pancreatic cancer and may have important implications for the management of this disease.

Introduction

Pancreatic cancer, one of the most lethal types of cancer worldwide, is a leading cause of cancer-associated mortality, with an overall 5-year survival rate of only 9% (1,2). Although the survival rates of the majority of cancer types have improved greatly, the diagnosis and treatment of pancreatic cancer remain difficult, and the total surgical resection rate of pancreatic cancer has not improved significantly over the past 20 years (3-5). Several mutational tumor suppressor genes and oncogenes, including SMAD family member 4 and tumor protein p53, have been revealed to contribute to the development of pancreatic cancer by promoting the transcription of downstream genes and affecting the cell cycle (6-8). Currently, a deep understanding of the underlying molecular mechanisms that promote pancreatic cancer remains elusive. Additionally, effective biomarkers and treatment are required to combat pancreatic cancer at an early stage (9). It is necessary to further elucidate the mechanism of pancreatic carcinogenesis and disease progression to reduce the mortality rate of pancreatic cancer (10).

P21 activated kinase 2 (PAK2) is a member of the Group I P21 activated kinases (PAK) family of serine/threonine kinases (11,12). The PAKs, effector molecules of Rac and cell division control protein 42 homolog, participate in various cellular signaling pathways, including cell adhesion, spreading, activation and other cell activities (13). PAKs phosphorylate numerous substrates, including those involved in cell survival, migration and apoptosis (14). Following activation by the rho guanosine 5’-triphosphatases, PAKs phosphorylate dozens of effector proteins to regulate cell activities, including mitogen-activated protein kinase signaling and cytoskeletal remodeling (15). PAKs also regulate cell homeostasis and the contraction process in various cellular environments (16).

Recent studies have indicated that PAK2 has shared and distinct functions in regulating cellular processes of different types of cells. PAK2 signaling modulates apoptosis, exhibiting anti-apoptotic and pro-apoptotic functions in an in vitro study (17,18). Previous studies have demonstrated that PAK2 is overexpressed in different types of human cancer, including ovarian cancer (19), gastric cancer (20), and head and neck cancer (21). Functional studies have also indicated that PAK2 contributes to several processes, including tumor cell proliferation, survival and invasion (22-24).
Previous studies have demonstrated that PAK2 serves a pivotal role in the progression of various tumors (17-24); however, the role of PAK2 in pancreatic cancer remains unclear. While PAK2 is ubiquitously expressed, it exhibits lower expression levels in the pancreas compared with other organs and tissues. Additionally, PAKs have been demonstrated to be associated with glucose homeostasis and the insulin signaling pathway in tissues, including the pancreas (25,26). A study has suggested that PAK2 is important in the activation of diverse pancreatic acinar cell signaling cascades and in the onset of early pancreatitis events (27). Therefore, the present study aimed to define the potential role of PAK2 in the pathogenesis of pancreatic cancer.

Materials and methods

Specimen collection and ethics statement. Following Institutional Review Board approval, the present retrospective study identified 82 patients [range. 40-70 years old, mean 56.2 years old, male=46 (56.1%) cases and female=36 (43.9%) cases] undergoing standard radical resection of pancreatic cancer at the Tianjin Nankai Hospital (Tianjin, China) between March 2011 and December 2015. The clinicopathologic features, including pTNM stage, tumor differentiation grade and so on were based on previous literature (20,21). The use of human samples and animals in the present study was approved by the Ethics Committee of the Tianjin Nankai Hospital. The present study was approved by Tianjin Nankai Hospital (Tianjin, China). Written informed consent to participate in the present study was provided by the participants.

Antibodies. Anti-PAK2 (1:200 dilution for immunohistochemistry and 1:1,000 dilution for western blotting; cat. no. ab76293; Abcam, Cambridge, UK), anti-β-actin (1:1,000 dilution; cat. no. ab8226; Abcam), anti-Ki-67 (1:1,000 dilution; cat. no. ab16667; Abcam), anti-proliferating cell nuclear antigen (PCNA; 1:500 dilution; cat. no. ab29; Abcam), anti-matrix metalloproteinase (MMP) 2 (1:1,000 dilution; cat. no. ab37150; Abcam) and anti-MMP9 (1:1,000 dilution; cat. no. ab38898; Abcam) were used in this study.

Immunohistochemistry. Samples of human pancreatic tissues were obtained from patients who underwent surgical resection at Tianjin Nankai Hospital. The fresh tissues were fixed with 4% paraformaldehyde at room temperature for 24 h. Paraffin-embedded tissue sections were cut 5 µm thick, deparaffinized, and rehydrated with xylene and graded alcohols. Following antigen retrieval [Boiling hot repair: Electric or raffinized, and rehydrated with xylene and graded alcohols. The presence of tan or brown particles in the cytoplasm indicated positive cells, and the percentage of positively stained cells was assessed as follows: Negative cells or <25% positive cells, score 0; 26-50% positive cells, score 1; 51-75% positive cells, score 2. Subsequently, the staining intensity was evaluated: Colorless, score 0; light yellow, score 1; deep yellow and tan, score 2; brown, score 3. The expression of PAK2 was calculated based on the aforementioned method (20,21), as follows: Staining index=staining intensity x tumor cell staining grade. Based on the staining index, low expression (0-2) and high expression (3-6) groups were classified. Two qualified expert pathologists, who were unaware of any clinical data of the patients, independently evaluated the result of each specimen.

Cell culture and transfection. PANCl and BxPC3 cells [The cell lines were purchased from the Beijing Xiehe Cell Resource Center (Beijing, China)] were cultured in H-Dulbecco's modified Eagle's medium (Gibco; Thermo Fisher Scientific, Inc., Waltham, MA, USA) and RPMI 1640 medium (Gibco, Waltham, Massachusetts, USA), respectively, supplemented with 10% fetal bovine serum (Gibco; Thermo Fisher Scientific, Inc.), at 37˚C in a humidified atmosphere with 5% CO₂ for 48 h. Plasmids (SH828063, Vige Biosciences Inc., Rockville, MD, USA) were transfected into cells by Lipofectamine® 2000. (Invitrogen; Thermo Fisher Scientific, Inc.). PAK2 was targeted using the following specific short hairpin RNA (shRNA) sequence: 5'-AAAA GCC CTT TTG TCA GCCAATCA-3'. A scrambled sequence was used as a negative control. The shRNAs (cat. no. #SH828063) were synthesized by ViGene Biosciences, Inc. (Rockville, MD, USA). A total of 100,000 cells/well in a 6-well plate were divided in two groups: A sh-PAK2 group transfected with shRNA (1.5 µg/ml) targeting PAK2 at 37˚C, and a negative control group transfected with the scrambled sequences, according to the manufacturer's protocol. Silencing efficiency was measured by reverse transcription-quantitative polymerase chain reaction (RT-qPCR) and western blotting after 48 h of transfection.

RT-qPCR. Total RNA was isolated by TRIzol® reagent (Invitrogen; Thermo Fisher Scientific, Inc.). RNA was reverse-transcribed using PrimeScript RT Reagent kit (Takara Bio, Inc., Otsu, Japan). qPCR was performed by Primer Mix Taq II (Takara Biotechnology Co., Ltd., Dalian, China). Primer sequences used were:

PARK2, forward 5'-ATCTTCCAGGCTCCTGACA-3', reverse 5'-TGAAGCTGCATCACTTCTTCTG-3'; and β-actin, forward 5'-CAGCTCACATGATGATGATC-3' and reverse 5'-AA GCCGCGCTTTGCACAT-3'. mRNA levels were quantified using the 2^{-ΔΔCq} method (28): The thermocycling conditions were as follows: 95˚C for 3 min in 1 cycle; 95˚C for 5 sec, 58˚C for 30 sec, and 72˚C for 30 sec in 35 cycles. To ensure the DNA production, a melting curve analysis was performed according to ABI StepOne system (Applied Biosystems; Thermo Fisher Scientific, Inc., Waltham, MA, USA). The relative gene expression was normalized to the internal β-actin control.
Western blot analysis. Cells and xenograft tumor tissues were lysed by RIPA lysis buffer (Beyotime Institute of Biotechnology, Haimen, China), and protein concentrations were measured by using the bicinchoninic acid Assay kit (BioSharp, Hefei, China). Protein samples (30-50 µg) were separated by 10% SDS-PAGE and transferred onto polyvinylidene difluoride membranes (EMD Millipore, Billerica, MA, USA), followed by blocking with Tris-buffered saline containing 0.2% Tween-20 and 5% fat-free milk at 37˚C for 40 min. Subsequently, membranes were incubated with primary antibodies at 4˚C overnight. After being washed three times, the biotinylated secondary antibody antibody-HRP (dilution, 1:1,000; cat. no., 00001-2; Wuhan Sanying Biotechnology Wuhan, China) was incubated for 1 h at room temperature and then washed again. At last the gray values were analyzed by using the Odyssey 3.0 software.

Cell proliferation and colony formation assays. For the cell proliferation assay, 2,000 cells (200 µl) were seeded in 96-well plates at 37˚C in 5% CO₂ and the density of cells was determined by MTT assays: After three days, the medium was removed and 20 µl MTT solution was added into each well at 37˚C for 4 h. Then, dimethyl sulfoxide (DMSO) was added (150 µl/well) into wells to solubilize the formazan crystals. This plate was incubated at room temperature for 10 min on a shaking table. The results were measured at a wavelength of 570 nm by spectrophotometry. The MTT assay results were indicated as optical density (OD) values by microplate reader. For the colony formation assay, cells were plated in 6-well plates at a density of 6,000 cells/well and grown for 14 days. The colonies were fixed with methanol and stained with 0.1% crystal violet at room temperature for 20 min. Images were then captured by a camera (EOS M100; Canon, Inc., Tokyo, Japan) and the colonies were subsequently extracted with 10% acetic acid and quantified using a microplate reader at a wavelength of 570 nm.

Wound healing assays. Cells (2x10⁵) were grown to confluent monolayers and a wound was made mechanically with a 20-µl pipette tip. Cells were then washed twice with PBS to remove cell debris, and complete culture medium was added to allow for wound healing. Images of the wound were captured using a microscope (Olympus BX43) at 24 h, and the extent of wound closure was measured.

Transwell assay. Cell invasion in response to PAK2 depletion was measured by Transwell assay. The upper surface of the Mateigel transwell filter was coated with Matrigel. The cells (1x10⁵) suspended in 150 µl serum-free medium were added to the chamber, then the chamber was placed into a 24-well plate containing complete medium. After 24 h of incubation at 37°C, the filter was taken out and Matrigel on the upper surface of the filter was removed using cotton swabs. Cells on the underside of the Transwell filter were then fixed with 4% paraformaldehyde for 25 min, stained with 0.1% crystal violet.
for 15 min and images were captured using a light microscope (magnification, x200; Olympus BX43). The dye was extracted with 10% acetic acid, and quantified using a microplate reader at a wavelength of 570 nm.

**Examination of tumor growth in mice.** All procedures for mouse care and surgery were approved by the Institutional Animal Care Committee of Tianjin Nankai Hospital. Nude BalB/c mice (6-8 weeks, 18-22 g) were purchased from Beijing Vital River Laboratory Animal Technology Co., Ltd. (Beijing, China) and mice were housed in pathogen-free animal facilities at 18-29˚C and randomly assigned (n=4 per group) into 2 groups according to the PANC-1 cell groups described above. The humidity was 40-70%. The mice had access to food and water with 12-h light/dark cycle. To examine tumor growth, PANC-1 cells (3x10^6) were injected subcutaneously into the right flank of female athymic nude mice, and tumor volumes were measured. After the mice were sacrificed, tumors were isolated, imaged and weighed. To investigate the association between PAK2 and tumor metastasis PANC-1 cells (1x10^5) were injected into the tail vein of mice (n=3 per group). After 4 weeks, metastasis in the lung was detected and the tumors were harvested. Subsequently, the protein expression levels of PAK2, PCNA and MMP2 in the tumors were tested by western blotting and/or immunohistochemistry in order to explore the reason for the difference in tumor volume between the two groups.

**Statistical analysis.** All data were analyzed with SPSS 22.0 software (IBM Corp., Armonk, NY, USA). The categorical data were analyzed by χ^2 method. Kaplan-Meier method was performed to estimate the prognosis of patients and the log-rank test was used to compare the two survival curves. Measurement data were presented as the means ± standard deviation, and a Student's t-test was used to compare two groups. P<0.05 was considered to indicate a statistically significant difference.

**Results**

**PAK2 is highly expressed in pancreatic cancer.** To explore the potential association between PAK2 and pancreatic cancer development, immunohistochemistry was conducted to detect the expression of PAK2 in samples from patients who underwent surgical resection (Fig. 1A). A total of 82 pancreatic cancer (Fig. 1A) and paracarcinoma (Fig. 1B) tissues were collected to detect the expression levels of PAK2 by immunohistochemistry. The positive rate of PAK2 in pancreatic ductal adenocarcinoma tissues was higher than that for 82 patients with pancreatic ductal adenocarcinoma.

<table>
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<th>High (n=42)</th>
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^aP<0.05. PAK2, P21 activated kinase 2; pTNM, pathological tumor-node-metastasis.
in paracarcinoma tissues ($\chi^2=6.942$, $P=0.008$). In addition, the association between PAK2 expression and clinical features of pancreatic cancer was analyzed. The results of the present study revealed that patients in the PAK2 high-expression group had a significantly lower overall survival rate compared with the patients in the low-expression group (Fig. 1C, $P<0.05$). In addition, patients with high PAK2 expression exhibited higher pTNM stage, tumor differentiation grade (20,21), proportion of lymph node metastasis and vascular invasion compared with the low-expression group (Table I). However, no significant associations between PAK2 expression and other pathological factors, including age, sex and tumor size, were identified (Table I). These data indicated that the patients in the PAK2 high-expression group had poor prognosis, suggesting that PAK2 may serve an important role in the development of pancreatic cancer.

**PAK2 promotes the proliferation of pancreatic cancer cells.** To gain mechanistic insights into the role of PAK2 in pancreatic cancer, PAK2-targeted shRNA was used to knockdown the expression of PAK2 in PANC1 and BxPC3 cells. As shown in Fig. 2A, PAK2-targeted shRNA could reduce the mRNA expression levels of PAK2 in these two types of pancreatic cells, as measured by RT-qPCR ($P<0.05$). Also, the shRNA-treated group exhibited reduced protein expression levels of PAK2 compared with the control group ($P<0.05$, Fig. 2B).

Colony formation assays were performed to assess the role of PAK2 in the proliferation of cancer cells. The present study revealed that shRNA-mediated PAK2 knockdown markedly decreased the number of PANC-1 and BxPC3 cell colonies formed compared with the control group ($P<0.05$, Fig. 3A and B). Ki-67 and PCNA are markers of proliferative cells, which may also reflect tumor malignancy. Therefore, the expression levels of Ki-67 and PCNA were detected to further assess the effects of PAK2 on the proliferation of PANC1 and BxPC3 cells. The results revealed that the expression levels of Ki-67 and PCNA were decreased in the PAK2 depletion group compared with the control group ($P<0.05$, Fig. 3C and D).

**PAK2 is important for the motility of pancreatic cancer cells.** Since abnormal cancer cell motility is a requirement for tumor metastasis, wound healing and Transwell assays were conducted to assess the role of PAK2 in the regulation of pancreatic cancer cell motility. The data demonstrated that shRNA-mediated depletion of PAK2 expression significantly inhibited the extent of wound closure in PANC1 and BxPC3 cells ($P<0.05$, Fig. 4A). In addition, PAK2 shRNA markedly compromised the...
invasion of pancreatic cancer cells through Matrigel-coated membranes, with cell numbers and the optical density value at 570 nm significantly decreased following PAK2 depletion (P<0.05, Fig. 4B). Additionally, MMP2 and MMP9 are closely associated with the occurrence and development of a number of types of tumor; therefore, the expression levels of MMP2 and MMP9 may reflect the malignancy degree of tumors, which was detected in control and PAK2 depletion groups. The expression of MMP2 and MMP9 was decreased in PAK2 shRNA-transfected PANC1 and BxPC3...
cells (P<0.05, Fig. 4C and D). Taken together, these results demonstrated that PAK2 may serve a pivotal role in pancreatic cancer cell invasion.

PAK2 depletion impairs pancreatic cancer growth in mice. To further investigate the role of PAK2 in pancreatic cancer cell proliferation in vivo, control or PAK2 shRNA-transfected...
Figure 5. PAK2 promotes pancreatic cancer growth in mice. (A) PAK2-shRNA- and scramble shRNA-transfected PANC1 cells were injected subcutaneously into athymic nude mice and tumor volumes were measured every 3 days. Images were captured on the last day. (B) Lung metastatic tumors were harvested after 4 weeks of feeding. Tumors in the PAK2-shRNA group were smaller compared with the control group. (C) Protein expression level analysis of PAK2 in the two groups using western blotting and immunohistochemistry. (D) Protein expression level analysis of PCNA and MMP2 for the two groups using immunohistochemistry. Magnification, x200. Data are reported as the means ± standard deviation (n=3). Arrows show lung tumor. *P<0.05, compared with the control. MMP2, matrix metallopeptidase 2; PAK2, P21 activated kinase 2; PCNA, proliferating cell nuclear antigen; shPAK2, short hairpin RNA specific to PAK2; shRNA, short hairpin RNA.
PAK2 serve distinct roles in hepatocyte growth factor-induced proliferation of PAK1 and PAK2 are clearly different (24). Furthermore, the protein expression levels of PAK2 in control and PAK2 shRNA-transfected tumors of mice were tested. Transfection with PAK2 shRNA could effectively decrease PAK2 expression in vivo (Fig. 5C). And the expression levels of PCNA and MMP2 were significantly reduced in the shRNA-transfected group, suggesting its low proliferative and metastatic capacity (P<0.05, Fig. 5D). These data confirmed that PAK2 may serve an important role in pancreatic tumorigenesis in vivo.

Discussion

The high mortality rate of pancreatic cancer is due to its poor prognosis and lack of targeted treatments. Knowledge of the mechanisms underlying this disease is limited and novel therapeutic targets of pancreatic cancer are required to improve treatment of this disease. The present study, to the best of our knowledge, revealed a novel role for PAK2 in pancreatic cancer development. Patients with high expression levels of PAK2 had poor prognosis and PAK2 could promote cancer cell proliferation and invasion, which suggested that PAK2 may serve an important role in the development of pancreatic cancer.

High expression levels of PAK2 have been reported in head and neck and gastric cancer, and PAK2 serves a key role in the cell survival pathway in these types of cancer (20,21). PAK2 is also involved in ovarian cancer progression (28). In melanoma cells, proliferation is inhibited by a microRNA targeting PAK2 (22). PAK2 also affects the homeobox protein cut-like 1/PAK2-p34/actin signaling pathways to influence the proliferation of ovarian cancer cells (29). Additionally, PAK2 regulates extracellular matrix proteins and further affects the migration of ovarian cancer cells (19). Although there is no proof of a link between PAK2 and MMPs, considering these studies, together with the findings of the present study, it is reasonable to speculate that PAK2 may be involved in pancreatic cancer development, and PAK2 may serve a critical role in cell proliferation and migration under certain circumstances.

PAK2 is a member of the Group I PAK family. In the present study, the role of PAK2 in the development of pancreatic cancer was examined. Other members of the PAK family, such as P21 activated kinase 1 (PAK1), in coordination with PAK2, regulate a variety of cell activities. In recurrent respiratory papilloma, PAK1 and PAK2 are activated to promote Ral1-mediated cyclooxygenase-2 expression (30). In the development of ovarian cancer, the expression and phosphorylation of PAK1 and PAK2 are clearly different (24). Furthermore, molecular mechanisms underlying the regulation of cancer cell invasion by PAK1 and PAK2 are also different (31). PAK1 and PAK2 serve distinct roles in hepatocyte growth factor-induced morphological responses (32). Whether PAK1 is also involved in the development of pancreatic cancer, as is the case for PAK2, needs to be precisely studied.

Although PAK2 exhibited positive expression in the normal pancreas, this study demonstrated that PAK2 served a vital role in pancreatic cancer. PAK2 depletion led to the inhibition of tumor cell proliferation and migration through various regulatory mechanisms. It has been reported that PAKs promote the proliferation of cancer cells through the AKT serine/threonine kinase 1 and Raf-mitogen-activated protein kinases pathways (33). PAK2 may phosphorylate multiple substrates; however, whether substrate phosphorylation influences cancer cell proliferation and invasion requires further exploration. A recent study reported that pyruvate kinase muscle isozyme M2 promotes pancreatic ductal adenocarcinoma invasion via phosphorylation of PAK2 (34). A previous study also demonstrated that PAK2 may phosphorylate caspase-7 to inhibit drug-induced breast cancer cell apoptosis (18). PAK2 may also phosphorylate c-Jun to promote the process of cell transformation (35). In addition, PAK2 could regulate rearrangement of the cytoskeleton, which is essential for the proliferation and migration of cancer cells, which suggests that PAK2 could regulate cytoskeleton remodeling to affect this process (36). And the study reported that PAK2 promotes gestational trophoblastic cell proliferation and invasion, providing evidence for a link between PAK2 and cell proliferation and invasion (36).

In conclusion, the present study demonstrated that PAK2 promoted cell proliferation and migration in pancreatic cancer by regulating related proteins, including PCNA, Ki67, MMP2 and MMP9. However, due to the heterogeneity of cancer cells, the potential of PAK2 as a target for the management of pancreatic cancer requires further investigation.

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Availability of data and materials

All data generated or analyzed during this study are included in this published article.

Authors' contributions

GWY, JRB and DPZ conceived and designed the experiments, performed the experiments, contributed the reagents/materials/analytical tools and wrote the paper. JRB analyzed the data.

Ethics approval and consent to participate

The use of human samples and animals in the present study was approved by the Ethics Committee of the Tianjin Nankai
Hospital. Written informed consent to participate in the present study was provided by the participants.

Patient consent for publication

All patients gave their consent for the publication of the present study.

Competing interests

The authors declare that they have no competing interests.

References


