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Upregulation of DAB2IP Inhibits Ras Activity and Tumorigenesis in Human Pancreatic Cancer Cells

Yifan Duan, MD, PhD[1,2], Xiaoyu Yin, MD, PhD[3], Xiaorong Lai, MD, PhD[4], Chao Liu, MD, PhD[5], Wenjing Nie, MD, MM[6], Dongfeng Li, MM[7], Zijun Xie, MD, PhD[2,8], Zijun Li, MD, PhD[2,9], and Fan Meng, MD, PhD[2,10]

Abstract

KRAS mutation-induced Ras activation plays an important role in the pathogenesis of pancreatic cancer, but the role of wild-type Ras and Ras GTPase-activating proteins remains unclear. The present study was designed to determine the expression spectra of Ras GTPase-activating proteins genes in pancreatic cancer cells, and the role of DAB2IP, a Ras GTPase-activating proteins gene, in the development and progression of pancreatic cancer. Following the analyses of the expression profiles of 16 Ras GTPase-activating proteins in 6 pancreatic cancer cell lines including Bxpc-3 (with wild-type KRAS), Capan-2, Sw1990, Aspc-1, CFPAC-1, and Panc-1 (with mutant KRAS) and 1 normal human pancreatic ductal epithelial cell line, H6C7, the expression of DAB2IP messenger RNA was further analyzed by quantitative real-time polymerase chain reaction. The role of DAB2IP in pancreatic cancer was further investigated *in vitro* and *in vivo* by upregulating DAB2IP in Bxpc-3 cells through transfection of DAB2IP into Bxpc-3 cells with recombinant lentivirus. The DAB2IP expression in pancreatic cancer cells and tissues with wild-type KRAS was significantly lower than that in cells and tissues with mutant KRAS ($P < .05$). In Bxpc-3 cells with wild-type KRAS, overexpression of DAB2IP decreased the expression of P-AKT and P-ERK and the Ras activity; increased the expression of P-JNK and caspase 3; inhibited cell proliferation, invasiveness, and migration; and increased the cell sensitivity to cetuximab. Overexpression of DAB2IP inhibited tumor progression in a mouse model. In conclusion, DAB2IP downregulates Ras activity in wild-type pancreatic cancer cells. Overexpression of DAB2IP decreases the Ras activity, inhibits cell proliferation, and increases sensitivity to cetuximab in wild-type pancreatic cancer cells. In conclusion, DAB2IP may serve as a potential molecular therapeutic target for the treatment of pancreatic cancer.

Keywords

RasGAP, DAB2IP, tumor suppressors, pancreatic cancer, Ras activity, Bxpc-3 cells

Abbreviations

ASK1, apoptosis signal-regulating kinase 1; EMT, epithelial–mesenchymal transition; GAP, GTPase-activating proteins; HCC, hepatocellular carcinoma; NF-κB, nuclear factor κB; OD, optical density; PAGE, polyacrylamide gel electrophoresis;

[1] Department of Huiqiao Building, Nanfang Hospital, Southern Medical University, Guangzhou, China
[2] Department of Gastroenterology, Guangdong General Hospital, Guangdong Academy of Medical Sciences, Guangzhou, China
[3] Department of Gastrointestinal Surgery, The First Affiliated Hospital, Sun Yat-sen University, Guangzhou, China
[4] Department of Oncology Medicine, Guangdong General Hospital, Guangdong Academy of Medical Sciences, Guangzhou, China
[5] Department of Pathology, Guangdong General Hospital, Guangdong Academy of Medical Sciences, Guangzhou, China
[6] The Third People's Hospital of Foshan, Foshan City, Guangdong Province, China
[7] Research Center of Medical Sciences, Guangdong General Hospital, Guangdong Academy of Medical Sciences, Guangzhou, China
[8] Department of Gastroenterology, The Third Affiliated Hospital, Sun Yat-sen University, Guangzhou City, Guangdong Province, China
[9] Guangdong Provincial Institute of Geriatrics, Guangzhou, China
[10] Department of Gastroenterology, The First Affiliated Hospital of Gannan Medical University, Ganzhou, China

Corresponding Author:
Zijun Li, Department of Gastroenterology, Guangdong General Hospital, Guangdong Academy of Medical Sciences, Guangdong Provincial institute of Geriatrics, Guangzhou City, Guangdong Province, China.
Email: zijunli2005@aliyun.com

PBS, phosphate buffered saline; PI3k, PI3-Kinase (PI3K)-AKT; qRT-PCR, quantitative real-time polymerase chain reaction; RasGAPs, Ras GTPase activating proteins; RBD, Ras-binding domain; SDS, sodium dodecyl sulfate

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Introduction

Pancreatic cancer is one of the most aggressive and malignant tumors. Due to the absence of effective approaches for early diagnosis or treatment intervention, pancreatic cancer is associated with very low surgical excision rate, extremely high mortality (almost 100%), and a very low overall 5-year survival rate (<5%).[1,2] Chemotherapy and radiation therapy are ineffective in the majority of patients, regardless of Kras mutation status.[1,2] Therefore, there is an urgent need for a better understanding of the pathogenesis of pancreatic cancer and identifying novel therapeutic targets for this deadly disease.

Several signaling pathways have been shown to play important roles in the pathogenesis of pancreatic cancer through modulating proliferation or apoptosis of cancer cells.[3,4] One of the most investigated is the Ras signaling pathway. In general, Ras alternates between GTP-bound (active) and GDP-bound (inactive) forms in a cycle regulated by Ras guanine nucleotide exchange factors and Ras GTPase activating proteins (RasGAPs).[5-7] Decreased expression of RasGAPs can also activate the Ras signaling pathway and cause abnormal cell biological behavior.[7] Oncogenic Ras mutants have alterations that affect intrinsic properties of the GTPase, which are insensitive to the action of RasGAPs.[8] Mutations of KRAS, which is 1 of 3 Ras genes, can cause deregulation of cell proliferation by acting through the RAS–RAF–MEK–ERK kinase pathway, while simultaneously activating a cascade of antiapoptotic signals through the PI3-Kinase (PI3K)-AKT pathway.[9-13] By comparison, the affinities of RasGAPs for wild-type Ras are much higher than that for Ras mutants.[8]

The RasGAPs superfamily includes 16 types: RASAL3, RASA2, RASA3, IQGAP2, IQGAP3, SYNGAP1, GAPVD1, IQGAP1, ARHGAP5, RASAL2, RASA4, G3BP1, NF1, DAB2IP, RASAL1, and RASA1.[7] Their association with carcinogenesis, cancer development, and progression has been one of the areas of intense research. Of these, DAB2IP, also called ASK-interacting protein-1, has been implicated as a tumor suppressor, and its low expression has been found in human liver and prostate cancers.[14,15] However, its role in pancreatic cancer and underlying mechanism remain to be elucidated. In the present study, we determined the expression spectra of RasGAPs for the first time, analyzed the expression of DAB2IP in pancreatic cancer cells and tissues, and explored its potential role and clinical significance in pancreatic cancer.

Results

Expression Spectra of RasGAPs in Pancreatic Cancer

We used quantitative real-time polymerase chain reaction (qRT-PCR) to analyze the messenger RNA (mRNA) levels of 16 RasGAPs in 6 different pancreatic cancer cell lines as well as 1 normal pancreatic H6C7 cell line. There were significant differences in DAB2IP expression among pancreatic cancer cells with wild-type KRAS, with 5 mutations in KRAS, and H6C7 cells ($P < .05$; Figure 1), with the relative mRNA levels (mean $\pm$ standard deviation [SD]) being 11.91 $\pm$ 1.40, 38.78 $\pm$ 1.49, and 87.02 $\pm$ 5.92 in the 3 types of cells, respectively. Specifically, significantly different expression patterns of DAB2IP were observed between pancreatic cancer cells with wild-type KRAS and those with mutant KRAS. According to the RasGAP expression spectra in pancreatic cancer cells observed in the present study and DAB2IP mRNA expression in pancreatic cancer cells and pancreatic ductal cells observed in our previous study[16] (Figure 1), DAB2IP was selected as a research focal point in the subsequent experiments of the present study.

Expression of DAB2IP in Pancreatic Cancer Tissues and Cells

Western blotting assay showed that DAB2IP protein expression levels were decreased in pancreatic cancer cells with wild-type KRAS expression, compared to cells expressing mutant KRAS and H6C7 cells, in our previous study.[16]

Immunohistochemistry analysis also showed that the DAB2IP expression level in pancreatic cancer tissues was significantly lower than that in adjacent tissues and normal pancreatic tissues (Figure 2). Among the 33 patients, the scores were 0, +, ++, and +++ in pancreatic cancer tissues for 1, 8, 23, and 1 patients, respectively, whereas the scores were +, ++, and +++ in adjacent tissues for 4, 8, and 21 patients, respectively. Among the 4 cases with normal pancreatic tissues, all were scored as +++ (Supplementary Table 2).

Sequencing of pancreatic cancer tissues revealed 26 (78.8%) of the 33 cases with KRAS gene mutations; the scores were +, ++, and +++ in cancer tissues for 4, 21, and 1 patients, respectively. Among the 7 KRAS wild-type patients, the scores were 0, +, and ++ in pancreatic cancer tissues for 1, 4, and 2 patients, respectively There was an association between DAB2IP expression and KRAS type in pancreatic cancer tissues (Supplementary Table 3).

Stable Overexpression of DAB2IP in Bxpc-3 Cells

We used qRT-PCR and Western blotting to assess the expression levels of DAB2IP after lentivirus transfection. As shown in Figure 3A and 3B, the DAB2IP expression was higher in Bxpc-3-psin-DAB2IP cells than that in Bxpc-3-psin-EF2 cells ($P < .05$), indicating a successful cell transfection with

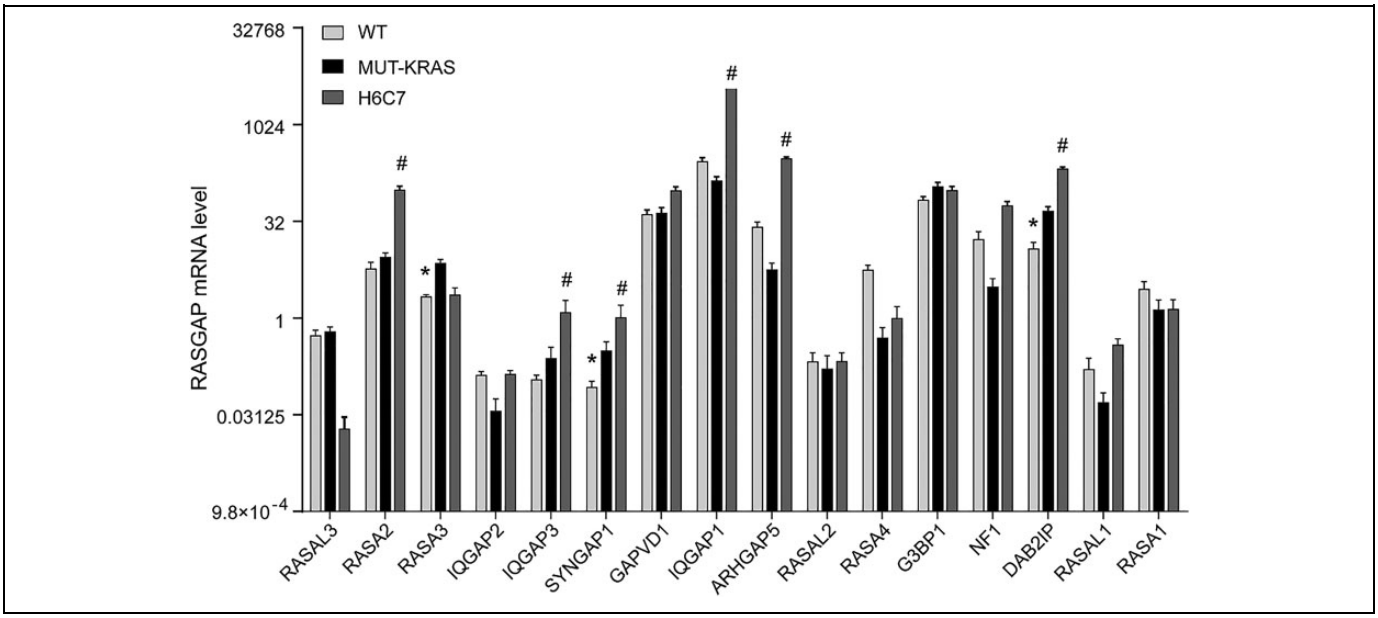

Figure 1. The messenger RNA (mRNA) expression levels of 16 Ras GTPase-activating proteins (GAPs) in 6 pancreatic cancer cell lines and a normal pancreatic ductal cell line. The RasGAPs superfamily includes 16 members: RASAL3, RASA2, RASA3, IQGAP2, IQGAP3, SYNGAP1, GAPVD1, IQGAP1, ARHGAP5, RASAL2, RASA4, G3BP1, NF1, DAB2IP, RASAL1, and RASA1. Quantitative real-time polymerase chain reaction (qRT-PCR) was used to analyze the RasGAPs mRNA levels in pancreatic cancer cells (expressing wild-type KRAS: Bxpc-3; expressing mutant KRAS: Capan-2, Sw1990, CFPAC-1, Aspc-1, Panc-1) and normal H6C7 cells. $^{\#}P < .05$, pancreatic cancer cells versus H6C7 cells; $^{*}P < .05$, pancreatic cancer cells with wild-type KRAS gene versus pancreatic cancer cells with a mutant KRAS gene.

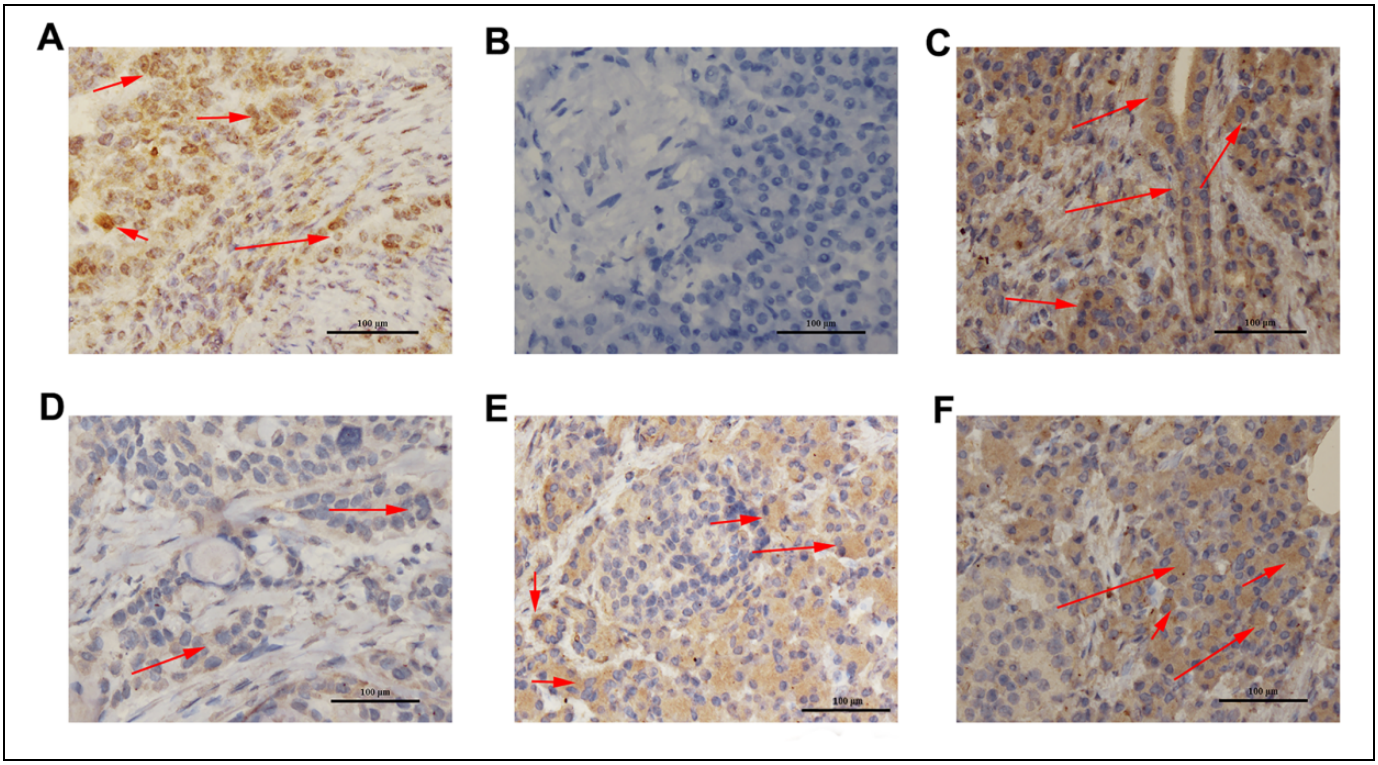

Figure 2. The expression levels of DAB2IP protein in pancreatic cancer tissues and controls, as analyzed by immunohistochemistry. (A) positive control (breast cancer); (B) negative control (pancreatic cancer, phosphate-buffered saline [PBS] was substituted for the primary antibody); (C) normal pancreatic tissue; (D) pancreatic cancer tissue with wild-type KRAS; (E) pancreatic cancer tissue with mutant KRAS; and (F) adjacent tissue. Magnification: ×400.

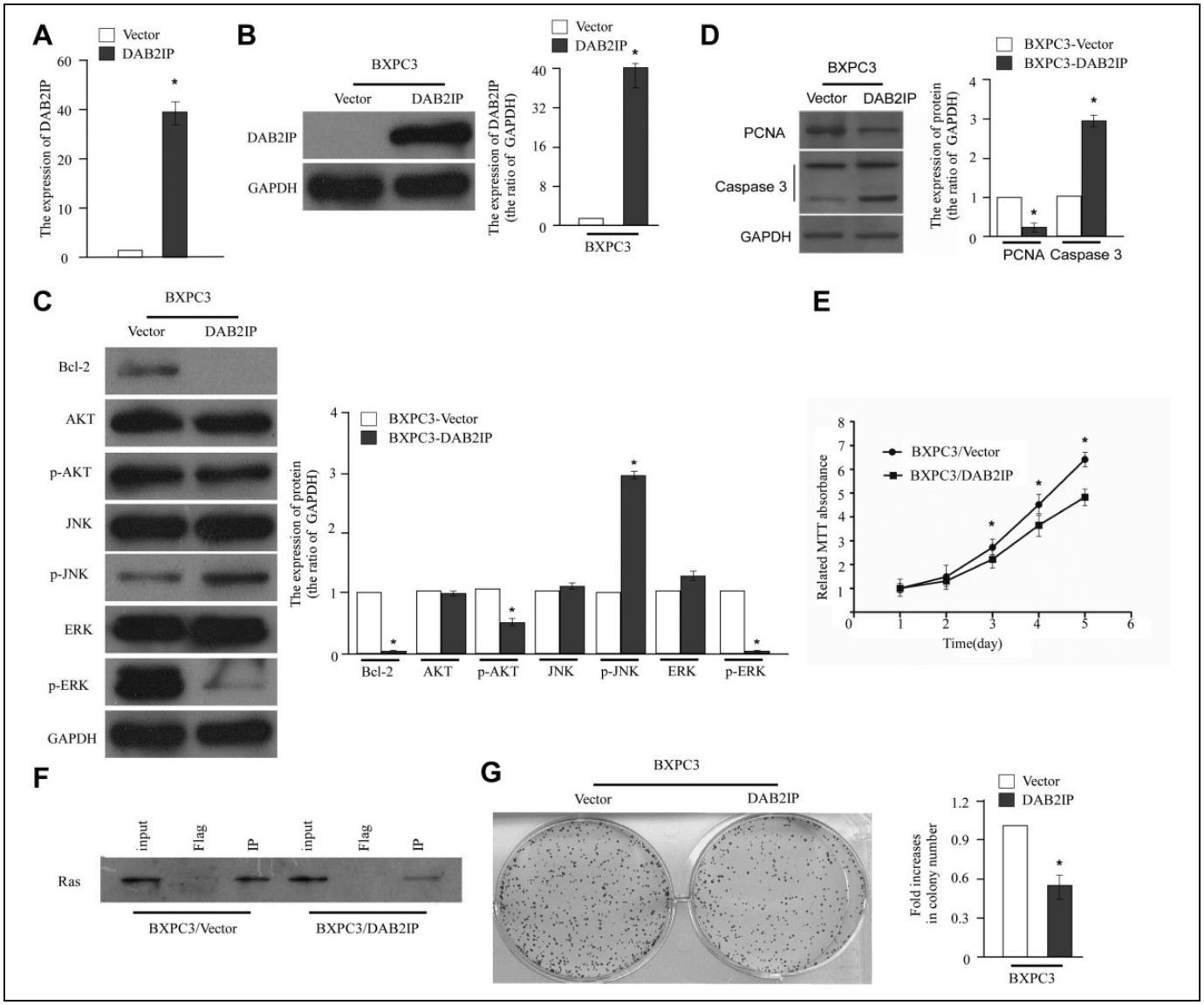

Figure 3. Validation of lentivirus transfection efficiency on Bxpc-3 cells and role of DAB2IP in the Ras signaling pathway. (A and B) The DAB2IP messenger RNA (mRNA) and protein expression levels were increased in wild-type KRAS pancreatic cancer cells after lentivirus transfection ($P < .05$) as assessed by quantitative real-time polymerase chain reaction (qRT-PCR) and Western blotting, respectively. (C and D), Overexpression of DAB2IP decreased the expression of P-AKT, P-ERK, and Bcl-2, coupled with increased expression of P-JNK and caspase 3 ($P < .05$), as assessed by Western blotting. (E) Overexpression of DAB2IP decreased the Ras activity ($P < .05$), as assessed by RAS-GTP pulldown assay; and (F and G) DAB2IP overexpression decreased the proliferation and colony formation of wild-type KRAS pancreatic cancer cells ($P < .05$), as assessed by 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2-H-tetrazolium bromide (MTT) and colony formation assays.

lentivirus harboring psin-EF2-DAB2IP, which remarkably enhanced the expression of DAB2IP.

Regulation of Ras Signaling Pathway and Ras Activity by Overexpression of DAB2IP

Changes in protein levels of Ras-GTP, which indicate RAS protein activity, and other components of the Ras signaling pathway such as P-AKT, P-ERK, and P-JNK were assessed in transfected Bxpc-3 cells by Western blotting. As shown in Figure 3C and E, the overexpression of DAB2IP in wild-type KRAS pancreatic cancer cells by transfection decreased the expressions of P-AKT, P-ERK, and the Ras activity, coupled with increased expression of P-JNK compared with control cells (all $P < .05$).

Effects of DAB2IP on Cell Proliferation and Colony Formation

The effects of DAB2IP on cell proliferation were detected by 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2-H-tetrazolium bromide (MTT) and colony formation assays. The MTT assay results showed that lower optical density (OD) values (decreased cell viabilities) were obtained in Bxpc3-psin-

DAB2IP cells (Figure 3F). As shown in Figure 3G, Bxpc3-psin-DAB2IP cells formed fewer colonies than Bxpc3-psin-EF2 cells. These data indicate that DAB2IP overexpression could decrease proliferation and colony formation of pancreatic cancer wild-type KRAS cells ($P < .05$).

Effects of DAB2IP on Cell Invasion and Migration

We also examined the change in migration capacity of Bxpc-3 cells after overexpression of DAB2IP by using Transwell cell invasion assay and wound healing assay. We found that Bxpc3-psin-DAB2IP cells exhibited impairment of migratory and invasive ability ($P < .05$; Figure 4A). As shown in Figure 4B, Bxpc3-psin-DAB2IP cells showed slower healing rate than Bxpc3-psin-EF2 cells ($P < .05$). These results suggest that overexpression of DAB2IP can decrease the migration capacity of Bxpc-3 cells wild-type KRAS expression.

Effects of DAB2IP on Cell Apoptosis and Cell Cycle

We next examined whether DAB2IP might affect cell cycle progression using a stable DAB2IP-transfected subline. The effects of DAB2IP on apoptosis and cell cycle progression were analyzed. We showed that more apoptotic cells were detected in stable DAB2IP-transfected Bxpc-3 cells, compared to the control cells ($3.17\% \pm 0.31\%$ vs $2.10\% \pm 0.26\%$, $P < .05$; Figure 4C), indicating that DAB2IP may be a proapoptotic factor. There were a higher percentage of Bxpc3-DAB2IP cells in the G0/G1 phase than in Bxpc3-Vector group. In contrast, there were less Bxpc3-DAB2IP in the S or G2/M phase cells than in Bxpc3-Vector cells ($P < .05$; Figure 4D). These results indicated that DAB2IP may elicit G0/G1 phase cell cycle arrest.

Effects of DAB2IP on Cell Sensitivity to Cetuximab

The Bxpc3-Vector cells and Bxpc3-DAB2IP cells were treated by stepwise exposure to increasing doses (1, 5, 10, 20, 50, 100, and 200 μg/mL) of cetuximab. The results from the MTT assay revealed that, with increasing concentration of cetuximab, the growth inhibition rates of Bxpc3-DAB2IP cells were greater than that of Bxpc3-Vector cells, demonstrating that the overexpression of DAB2IP enhanced the sensitivity of the pancreatic cancer cells with wild-type KRAS to the cetuximab, compared to the control cells ($P < .05$; Figure 5A).

Nude Mouse Tumorigenicity Assay

The nude mouse tumorigenicity assay showed that the tumor volumes in Bxpc3-DAB2IP group were significantly smaller than that in the Bxpc3-Vector group ($P < .05$; Figure 5B). The tumor growth in Bxpc3-DAB2IP + cetuximab group was also significantly smaller than the Bxpc3-Vector + cetuximab group ($P < .05$; Figure 5C and D). These results suggested that overexpression of DAB2IP inhibits tumor progression and enhances the sensitivity to cetuximab *in vivo*, as seen *in vitro*.

Discussion

Several recent studies have investigated the expression patterns of various RasGAPs in different cancers.[5,6,8] It is reported that decreased expression of RasGAPs activates the Ras signaling pathway and causes abnormal cell biological behavior in recent studies.[5,6,8] In fact, abnormal RasGAP expression can stimulate the RAS signaling molecules and lead to increased survival, invasion, and migration of cancer cells. Although they are insensitive to the action of RasGAPs, oncogenic Ras mutants retain the ability to bind them in the GTP-bound state. To date, the role of RasGAP in the progression of pancreatic cancer remains to be elucidated.

DAB2IP is one of the 16 RasGAPs and interacts with the tumor suppressor DAB2/DOC2 or the apoptosis signal-regulating kinase 1 (ASK1) in tumor necrosis factor-mediated JNK/P38 MAPK pathway.[17,18] Analysis of DAB2IP has demonstrated that DAB2IP exhibits RasGAP activity *in vivo* and *in vitro*.[19] Additionally, DAB2IP is frequently downregulated in metastatic prostate cancer and is therefore implicated as a prognostic risk factor for aggressive prostate cancer.[15,20,21] Furthermore, the cells with DAB2IP knockdown are resistant to radiation-induced apoptosis. Studies by Wu *et al*[22] also suggest that loss of DAB2IP expression in castration-resistant prostate cancer cells signifies their chemoresistance. Studies by Calvisi *et al*[14] show that, in the absence of Ras mutations, downregulation of DAB2IP is found in all human hepatocellular carcinoma (HCC) samples. Low levels of DAB2IP are detected mainly in an HCC subclass with poor survival, suggesting that DAB2IP levels are associated with tumor aggressiveness. These results suggest that selective suppression of DAB2IP results in unrestrained activation of Ras signaling in the presence of wild-type RAS in HCC.[14,23]

In the present study, we defined the expression spectra of the Ras GAP pancreatic cancer cells and normal human pancreatic ductal epithelial H6C7 cells for the first time. The results showed that DAB2IP expression was significantly lower in pancreatic cancer cells with wild-type KRAS. These results are consistent with our previous report which demonstrated that mutant-type KRAS tumors have increased active Ras protein expression and abnormal Ras signaling, and a correlation exists between the low expression and/or loss of function of inhibitors of Ras gene expression such as RasGAPs.[16] In a previous study, qRT-PCR was used to analyze DAB2IP mRNA in 4 types of pancreatic cancer cells and normal human pancreatic ductal epithelial cells.[16] However, in the present study, we revealed the differences in DAB2IP expression among 5 types of pancreatic cancer cells with wild-type or mutant KRAS expression.

The PI3K-AKT signaling pathway plays a central role in modulating cell proliferation, survival, and motility.[21,24-27] In addition to promoting cell proliferation and survival, the PI3K-AKT pathway can also influence apoptotic cell death machinery.[24-26] DAB2IP promotes apoptosis by activating the ASK1, an upstream inducer of JNK and P38 MARK proteins.[28]

In the present study, forced overexpression of DAB2IP in wild-type KRAS pancreatic cancer cells decreased the

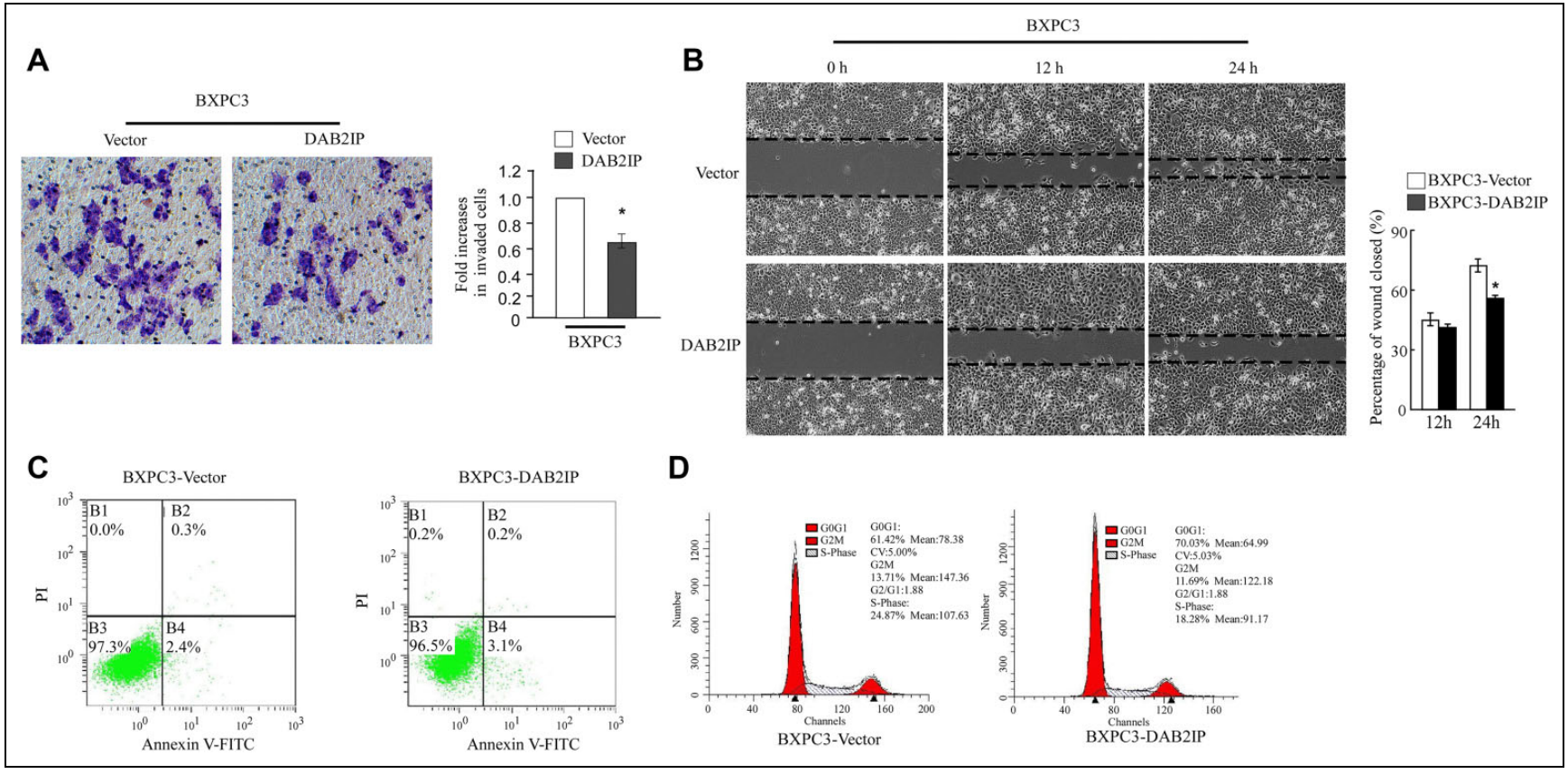

Figure 4. Effects of DAB2IP overexpression on cell invasion, migration, apoptosis, and cell cycle. (A and B) Overexpression of DAB2IP weakened the invasion and migration capacity of Bxpc-3 cells ($P < .05$), as assessed by Transwell cell invasion and wound healing assays. (C and D) Overexpression of DAB2IP increased cell apoptosis and the percentage of cells in the G0/G1 phase ($P < .05$), as assessed by Annexin V/propidium iodide assay.

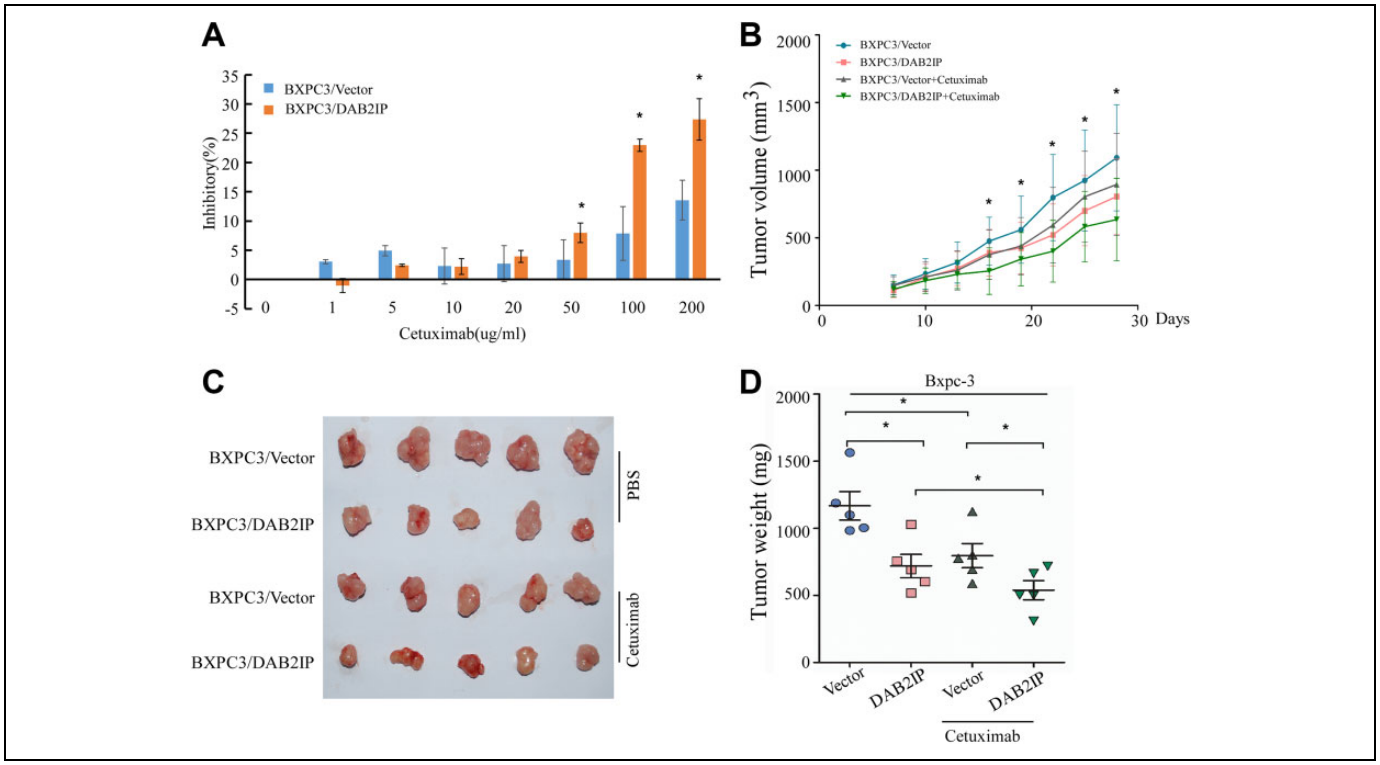

Figure 5. Effects of DAB2IP overexpression on cell sensitivity to cetuximab *in vitro* and on tumor growth *in vivo*. (A) Compared to the control Bxpc3-Vector cells, Bxpc3-DAB2IP + pancreatic cancer cells were more inhibited by cetuximab at various doses (50, 100, and 200 μg/mL). Overexpression of DAB2IP enhanced the sensitivity of the pancreatic cancer cells with wild-type KRAS to cetuximab, compared to the control cells ($P < .05$), as assessed by 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2-H-tetrazolium bromide (MTT) assay. (B-D) The tumor volume and weight were smaller in Bxpc3-DAB2IP + cetuximab group than in the Bxpc3-Vector + cetuximab group ($P < .05$), as assessed by nude mouse tumorigenicity assay. B refers to how the tumor volume in the Bxpc3-DAB2IP + cetuximab group was inhibited more than that in the control Bxpc3-Vector + cetuximab group, as well as inhibited more in the Bxpc3-DAB2IP group compared to the Bxpc3-Vector group ($P < .05$).

expression of P-AKT and P-ERK, and Ras activity, coupled with increased expression of P-JNK. It also decreased proliferation and enhanced apoptosis of pancreatic cancer Bxpc-3 cells with wild-type KRAS expression, indicating that DAB2IP may be a proapoptotic factor capable of potentiating intrinsic apoptotic pathways. Overexpression of DAB2IP decreased the migration capacity of Bxpc-3 cells.

Cell cycle checkpoint is a defense mechanism that allows cells to repair genetic lesions following DNA damage.[29,30] In the present study, we showed that DAB2IP could elicit G0/G1 phase cell cycle arrest. Annexin V/PI analysis showed that fewer viable cells were detected in stable DAB2IP-transfected Bxpc-3 cells. In addition, the overexpression of DAB2IP enhanced the sensitivity of the pancreatic cancer cells with wild-type KRAS to cetuximab. The *in vivo* nude mouse tumorigenicity assay also demonstrated that DAB2IP overexpression inhibited tumor proliferation and progression, as seen *in vitro*.

The epithelial–mesenchymal transition (EMT) is a dynamic cellular process thought to support cancer metastasis by promoting invasion. It has been confirmed that, during EMT, levels mesenchymal markers such as vimentin, twist, and fibronectin are increased with a concomitant reduction in membrane-bound E-cadherin. DAB2IP is able to suppress the proliferation, EMT, invasion, and metastasis of colorectal cancer cells.[31] Min *et al*[21] further demonstrated that a point mutant in the domain of DAB2IP leads to reduce the inhibitory effects on invasion and EMT, suggesting that nuclear factor (NF)-κB mediates these phenotypes in response to DAB2IP loss. In the present study, we revealed that overexpression of DAB2IP could reduce the proliferation, migration capacity, and apoptosis of pancreatic cancer cells with wild-type KRAS. Nevertheless, its relationship with EMT deserves warrants investigation.

The present study identified DAB2IP as a critical molecule in the process of pancreatic cancer among numerous target genes. While Ras and NF-κB are known to be activated in advanced cancer, the genetic alterations that confer their activation are largely unknown. As such genes may suppress both Ras and NF-κB in cancer and downstream proteins have been proven difficult to target directly, these findings may raise the question as to whether upregulation of DAB2IP may affect primary tumors and metastatic lesions.

In summary, the expression of DAB2IP was low in pancreatic cancer cells and tissues, and correlated with the type of KRAS gene, the presence of PNI and clinical stage of the disease. DAB2IP could modulate the proliferation, migration capacity, and apoptosis of pancreatic cancer cells with wild-type KRAS gene via Ras-Raf-ERK, PI3K-Akt, and ASK1-JNK

signaling pathways. Overexpression of DAB2IP also enhanced the sensitivity to cetuximab and inhibited tumor progression *in vitro* and *in vivo*, thus indicating that DAB2IP may serve as a potential promising molecular therapeutic target for pancreatic cancer.

Materials and Methods

Research Involving Human Participants

Ethical approval. This study was approved by the Institutional Review Board and Ethics Committee at Guangdong General Hospital. All procedures involving human participants and animals were performed in accordance with the ethical standards of the Institutional Review Board and with the 1964 Helsinki declaration and its later amendments or comparable ethical standards. The approval numbers (2010132A, 2012080H) were provided by the ethical board, and all written informed patient consent and permission were obtained.

Cell Lines and Human Tissue Samples

The 6 pancreatic cancer cell lines (wild-type KRAS: Bxpc-3; mutant KRAS: Capan-2, Sw1990, Aspc-1, CFPAC-1, and Panc-1) were purchased from the American Type Culture Collection (Rockville, Maryland) and the Shanghai Cell Bank (Shanghai, China). A normal human pancreatic ductal epithelial cell line, H6C7, was kindly provided by Professor Zhang Shineng from Sun Yat-sen University, Guangzhou, China.

Paraffin-embedded specimens of pancreatic cancer tissues and adjacent noncancerous tissues (33 pairs) were collected in Guangdong Province People's Hospital and The First Affiliated Hospital of Sun Yat-sen University between 2008 and 2014. Patients (23 males and 10 females; aged from 37 to 73 years, median 62 years) did not receive any preoperative chemotherapy or radiotherapy or immunobiological therapy for pancreatic cancer. The Diagnosis and Treatment Standard of Pancreatic Cancer[32] was used as a reference for staging pancreatic cancer. Genomic DNA was extracted and used for detection of KRAS mutation at common mutation points by PCR. Sequencing of pancreatic cancer tissues ($n = 33$) revealed KRAS gene mutations in 25 cases and wild-type KRAS expression in 8 cases.

Quantitative Real-Time PCR for Detecting mRNA Expression of RasGAP

Total RNA was extracted from pancreatic cancer cells and H6C7 cells, using TRIzol reagent (Invitrogen, Carlsbad, California). The primers for amplification of the 16 members of RasGAP superfamily and GAPDH (control) were designed by Shanghai Jierui Co (Shanghai, China). The sequences of the DAB2IP primers were as follows:

forward: 5′-CCTGGACGATGTGCTCTATG-3′; and reverse: 5′-TCTTCTTCTTCTTGTCGGTCTC-3′.

The primer sequences used in qPCR for the remaining RasGAP superfamily members are listed in Supplementary Table 1.

The qRT-PCR analysis was performed using an ABI PRISM 7500 Quantitative PCR system (Applied Biosystems, Foster City, California). Amplification conditions were as follows: 95°C for 10 minutes, followed by 50 cycles of 95°C for 30 seconds, 58°C for 30 seconds, and 72°C for 30 seconds. Each sample was analyzed in triplicate, and the amount of product was normalized relative to that of GAPDH. Quantitative values were calculated according to the formula: Quantitative values $= 2-\Delta CT$, in which the ΔCT value for each GAP was calculated by subtracting the average CT value for the target gene from the average CT value for the GAPDH gene.

Western Blot Analysis for Detecting Protein Expression of DAB2IP and Components of Ras Signaling Pathway

Total proteins were extracted from pancreatic cancer cells and H6C7 cells using RIPA buffer (Shanghai Biocolor BioScience Technology Company, Shanghai, China) containing protease and phosphatase inhibitors. The samples of equal total proteins were subjected to sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE), and the proteins were transferred to polyvinyl difluoride membranes (Millipore, Shanghai, China). Polyclonal anti-DAB2IP (1:2000; Abcam, Shanghai, China) and β-actin (1:3000; Bioworld, Nanjing, China) antibodies were used as primary antibodies, and horseradish peroxidase (HRP)-conjugated goat antirabbit antibody (1:2000; Cell Signalling Technology, Shanghai, China) was used as the secondary antibodies. Immunoreactive proteins were visualized using an enhanced chemiluminescence (ECL; Invitrogen) system, and the band density was analyzed by BandScan version 5.0. The expression levels of components of the Ras signaling pathway, including AKT (1:1500, Cat. No. 9272; CST, Shanghai, China), ERK (1:1500, Cat. No. BM4326; Santa Cruz, beijing, China), JNK (1:1500, Cat. NoBA1219; Boster, hubei, China) and their active P-AKT (1:1500, Cat. No.4060 S; CST), P-ERK (1:1500, Cat. No. BM3950; Santa Cruz, beijing, China), and P-JNK (1:1000, Cat. No.4668; CST) as well as Ki-67 (1:1500, Cat. No. 2714 -1; EPIT MICS, shanghai, China), caspase-3 (1:1500, Cat. No. SC-7272; Santa Cruz, beijing, China), and Bcl-2 (1:1500, Cat. No. BM0200) were also monitored. GADPH was used as an internal control (Cat. No. BM1623; Boster, hubei, China).

Immunohistochemistry for Detecting Protein Expression of DAB2IP

Paraffin-embedded tissue specimens were sectioned (4-μm thick) and slides prepared using standard techniques. Mounted tissue sections were baked at 65°C for 3 hours, deparaffinized in xylene, and rehydrated through graded alcohols. Antigens were retrieved by heating in 1 μμ sodium citrate (pH 6.0) in a pressure cooker at 100°C for 3 minutes, followed by incubation in 3% H_2O_2 for 10 minutes at room temperature to destroy

endogenous peroxidase activity. Nonspecific staining was blocked by incubation in 10% sheep serum for 10 minutes. Tissue sections were incubated with anti-DAB2IP (1:200; Abcam) primary antibodies overnight at 4°C, followed by HRP-conjugated incubation with an EnVision goat antirabbit antibody secondary antibody (Shanghai gene company, China) for half an hour at room temperature. Sections were stained with diaminobenzene and hematoxylin, dehydrated through graded alcohols and xylene, and then mounted. Human breast cancer specimens were used as the positive control, and phosphate buffered saline (PBS) was substituted for the primary antibody, as the negative control. Positively stained cells exhibited clear cell structure, accurate positioning of positive granules, and a distinct contrast to the background staining. Positive staining intensity (A) was graded as follows: 0 (no staining), l (weak staining, light yellow), 2 (moderate staining, brown yellow), and 3 (brown). Positive staining cell count (B) was scored as follows: 1 (<1/3), 2 (1/3–2/3), 3 (≥2/3). The degree of positive staining (A × B) was represented as follows: A × B = 0: (-), A × B = 1 – 2: (+), A × B = 3–4: (++), A×B = 6–9: (+++). Data were obtained for 5 to 10 randomly selected high magnification fields.

Stable Transfection of DAB2IP Into Bxpc-3 Cells With Recombinant Lentivirus

The DAB2IP expression construct was generated by subcloning PCR-amplified full-length human DAB2IP complementary DNA into the pSin-EF2-puro lentiviral vector (Addgene, beijing, China) by *EcoR*I and *Nhe*I. pSin-EF2-puro-DAB2IP or the empty lentiviral vector and 2 packaging plasmids *pM2.G* and *psPAX2* (Addgene, beijing, China) were cotransfected into 293 T cells using the calcium phosphate transfection method, as described previously. After infection, puromycin (0.5 g/mL; Sigma, shanghai, China) was used to select stably transfected cells. The DAB2IP mRNA and protein expression levels were examined by RT-PCR and Western blotting analyses, respectively. The PCR and sequencing analyses confirmed that the DAB2IP gene sequence was consistent with the sequence in GenBank (NCBI, NM_138709). Stably transfected Bxpc-3-psin-EF2-DAB2IP and Bxpc-3-psin-EF2 cell lines were constructed, packaged, and purified by Laura Biotech Company (Guangzhou, China).

Mutational Analysis

Genomic DNA was extracted from stably transfected Bxpc-3-psin-EF2-DAB2IP and Bxpc-3-psin-EF2 cells using QIAamp DNA FFPE Tissue kit (Qiagen, Shanghai, China) and used as a template for amplification of the KRAS common mutations point by PCR with the Premix Ex Taq Hot Start Version kit (TaKaRa, Dalian, China). The PCR products were purified and sequenced. The PCR analysis was performed using the ABI Big Dye Terminator v3.1 kit (Applied Biosystems, San Ramon, California). The PCR products were rinsed, predenatured, and transferred to 96-well plates for direct sequencing using an ABI 3100 Genetic Analyzer (Applied Biosystems).

Ras Activation Assay

The Ras activity assay was carried out in stably transfected Bxpc-3-psin-EF2-DAB2IP and Bxpc-3-psin-EF2 cells using a Ras Activation Assay Kit (Millipore). Active RAS was pulled down with Raf-1 Ras-binding domain (RBD) agarose beads. The pull-down proteins were subjected to SDS-PAGE and immunoprecipitation with anti-Ras antibody (CST). Active RAS was pulled down with Raf-1 RBD agarose beads. The pulled-down proteins were subjected to SDS-PAGE and immunoprecipitation with anti-Ras antibody. The Ras activation was monitored using a GST-fusion protein containing the RBD of Raf1.

MTT Assay

Cells with upregulated DAB2IP, negative control, and untreated control cells were seeded (1×10^4 cells per well) onto 96-well plates and incubated at 37°C for 24 hours. In parallel, a blank control group containing only medium without cells was used. Each sample was analyzed 6 times. Cells were incubated for 24, 48, 72, and 96 hours, respectively. At the end of each incubation period, the MTT solution (5 mg/mL, 20 μL/well) was added and further incubated at 37°C for 4 hours. After the medium was carefully aspirated, 150 μL of dimethyl sulfoxide was added. Following shaking for 10 minutes, the OD was determined at 490 nm using a microplate reader (Thermo Scientific, Beijing, China).

Colony Formation Assay

For colony formation assay, the cells were plated in 6-well plates at a density of 500 cells per well and transfected. Then, the cells were maintained in RPIM-1640 containing 10% fetal bovine serum for 10 days. Colonies were fixed using methanol, stained with 0.1% Crystal violet for 10 minutes, and then photographed.

Cell Invasion Assay

Transwell cell invasion assay was carried out using Growth Factor Reduced Matrigel invasion chambers with 8.0-mm polycarbonated filters (BD Biosciences, Shanghai, China). In brief, 2.5×10^4 cells were seeded on chamber plates and cultured in RPMI 1640 medium. After a 22-hour incubation at 37°C in a humidified incubator with 5% CO_2, nonmigratory cells on the upper surface of the filter were removed by wiping with a cotton swab. The invasive cells that penetrated through pores and migrated to the underside of the membrane were stained with Crystal violet and counted and photographed under a microscope.

Wound Healing Assay

A linear wound was made by scraping with a Pasteur pipette across the confluent cell layer 24 hours after treatment by mitomycin C. The cells were washed twice to remove detached cells and debris. Then, fresh medium was added, and the size of the wound was measured at the indicated times.

Annexin V/Propidium Iodide Assay for Apoptosis and Cell Cycle

The cells were harvested, and a single-cell suspension was prepared. The cells were then washed twice, resuspended at 1 to 2×10^6 cells/mL, and transferred in 1 mL into a 15-mL polypropylene, V-bottomed tube, to which 3 mL cold absolute ethanol was added. The cells were fixed for at least 1 hour at 4°C and washed twice with PBS. Propidium iodide staining solution (1 mL) was added to the cell pellet and mixed well, and 50 μL of RNase A stock solution was then added and incubate for 3 hours at 4°C. The samples were analyzed by flow cytometry (BD Company, Franklin Lakes, New Jersey).

Nude Mouse Tumorigenicity Assay

Stably transfected Bxpc-3-psin-EF2-DAB2IP and Bxpc-3-psin-EF2 cells were obtained. These cells were washed with PBS and resuspended in PBS at a concentration of 1×10^7 cells/mL. The cell suspension (0.1 mL) was subcutaneously injected into both sides of the flank of the same nude mice (12 nude mice per group). After tumor formation, 6 mice were randomly selected from each group and received increasing doses (1, 5, 10, 20, 50, 100, and 200 μg/mL) of cetuximab treatment. Tumor growth was monitored every 3 days. After 30 days, these mice were killed and examined for the growth of subcutaneous tumors.

Statistical Analysis

The experimental data were presented as mean $\pm$ SD. All statistical analyses were performed using the SPSS version 13.0 statistical software (SPSS Inc, Beijing, China). Differences in DAB2IP mRNA and protein levels between the pancreatic cells and H6C7 were assessed by 1-way analysis of variance, and the differences between groups were assessed using the Student-Newman-Kuels test. Value of $\alpha = .05$ (2-sided) was set as the difference level. Differences in DAB2IP protein expression levels between the pancreatic cancer tissues and the adjacent tissues were assessed using 2 related sample's Wilcoxon tests. The relationships between DAB2IP protein expression and clinical pathological parameters were analyzed using 2 independent samples in Wilcoxon tests. Value of $P < .05$ was considered statistically significant.

Authors' Note

Yifan Duan and Xiaoyu Yin contributed equally to this work, and both are co-first authors. Zijun Xie, Zijun Li and Fan Meng are also affiliated with Department of Gastroenterology, Guangdong General Hospital, Guangdong Academy of Medical Sciences, Guangzhou, China.

Declaration of Conflicting Interests

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ORCID iD

Zijun Li https://orcid.org/0000-0002-3029-5001

Supplemental Material

Supplemental material for this article is available online.

References

1. Hidalgo M. Pancreatic cancer. *N Engl J Med*. 2010;362:1605-1617.
2. Rossi ML, Rehman AA, Gondi CS. Therapeutic options for the management of pancreatic cancer. *World J Gastroenterol*. 2014;20(32):11142-11159.
3. Di Marco M, Astolfi A, Grassi E, et al. Characterization of pancreatic ductal adenocarcinoma using whole transcriptome sequencing and copy number analysis by single-nucleotide polymorphism array. *Mol Med Rep*. 2015;12(5):7479-7484.
4. Sano M, Driscoll DR, DeJesus-Monge WE, et al. Activation of WNT/β-catenin signaling enhances pancreatic cancer development and the malignant potential via up-regulation of Cyr61. *Neoplasia*. 2016;18(12):785-794.
5. Hennig A, Markwart R, Esparza-Franco MA, et al. Ras activation revisited: role of GEF and GAP systems. *Biol Chem*. 2015;396(8):831-848.
6. Grewal T, Koese M, Tebar F, et al. Differential regulation of RasGAPs in cancer. *Gen Can*. 2011;2:288-297.
7. King PD, Lubeck BA, Lapinski PE. Nonredundant functions for Ras GTPase-activating proteins in tissue homeostasis. *Sci Signal*. 2013;26(6):264.
8. Maertens O, Cichowski K. An expanding role for RAS GTPase activating proteins (RAS GAPs) in cancer. *Adv Biol Regul*. 2014;55:1-14.
9. Ciardiello F, Tortora G. Targeting RAS signaling pathway as a potential therapeutic target in the treatment of colorectal cancer. *J Cell Physiol*. 2018;233(3):2058-2066.

10. Charette N, Vandeputte C, Stärkel P. Ras in digestive oncology: from molecular biology to clinical implications. *Curr Opin Oncol*. 2014;26(4):454-461.
11. Bournet B, Buscail C, Muscari F, et al. Targeting KRAS for diagnosis, prognosis, and treatment of pancreatic cancer: hopes and realities. *Eur J Cancer*. 2016;54:75-83.
12. Vincenzi B, Cremolini C, Sartore-Bianchi A, et al. Prognostic significance of *K-Ras* mutation rate in metastatic colorectal cancer patients. *Oncotarget*. 2015; 6(31): 31604-31612.
13. Pedersen K, Bilal F, Bernadó Morales C, et al. Pancreatic cancer heterogeneity and response to Mek inhibition. *Oncogene*. 2017; 36(40):5639-5647.
14. Calvisi DF, Ladu S, Conner EA, et al. Inactivation of Ras GTPase-activating proteins promotes unrestrained activity of wild-type Ras in human liver cancer. *J Hepatol*. 2011;54: 311-319.
15. Yang C, He H, Zhang T, et al. Decreased DAB2IP gene expression, which could be induced by fractionated irradiation, is associated with resistance to γ rays and α particles in prostate cancer cells. *Mol Med Rep*. 2016;14(1):567-573.
16. Duan YF, Li DF, Liu YH, et al. Decreased expression of DAB2IP in pancreatic cancer with wild-type KRAS. *Hepatobiliary Pancreat Dis Int*. 2013;12:204-209.
17. Wang B, Gu Q, Li J. DOC-2/DAB2 interactive protein regulates proliferation and mobility of nasopharyngeal carcinoma cells by targeting PI3K/Akt pathway. *Oncol Rep*. 2017;38(1):317-324.
18. Ji W, Li Y, Wan T, et al. Both internalization and AIP1 association are required for tumor necrosis factor receptor 2-mediated JNK signaling. *Arterioscler Thromb Vasc Biol*. 2012;32(9): 2271-2279.
19. Liu L, Xu C, Hsieh JT, et al. DAB2IP in cancer. *Oncotarget*. 2016;7(4):3766-3776.
20. Kong Z, Raghavan P, Xie D, et al. Epothilone B confers radiation dose enhancement in DAB2IP gene knock-down radioresistant prostate cancer cells. *Int J Radiat Oncol Biol Phys*. 2010;78: 1210-1218.
21. Min J, Zaslavsky A, Fedele G, et al. An oncogene-tumor suppressor cascade drives metastatic prostate cancer by coordinately activating Ras and nuclear factor-kappaB. *Nat Med*. 2010;16: 286-294.
22. Wu K, Xie D, Zou Y, et al. The mechanism of DAB2IP in chemoresistance of prostate cancer cells. *Clin Cancer Res*. 2013;19: 4740-4749.
23. Zhang X, Li N, Li X, et al. Low expression of DAB2IP contributes to malignant development and poor prognosis in hepatocellular carcinoma. *J Gastroenterol Hepatol*. 2012;27(24): 1117-1125.
24. Schmidt JW, Wehde BL, Sakamoto K, et al. Stat5 regulates the phosphatidylinositol 3-kinase/Akt1 pathway during mammary gland development and tumorigenesis. *Mol Cell Biol*. 2014; 34(7):1363-1377.
25. Murthy D, Attri KS, Singh PK. Phosphoinositide 3-kinase signaling pathway in pancreatic ductal adenocarcinoma progression, pathogenesis, and therapeutics. *Sign Front Physiol*. 2018; 9:335.
26. Wu T, Song H, Xie D, et al. Silencing of ASPP2 promotes the proliferation, migration and invasion of triple-negative breast cancer cells via the PI3K/AKT pathway. *Int J Oncol*. 2018;52(6): 2001-2010.
27. Zhao S, Jiang Y, Zhao J, et al. Quercetin-3-methyl ether inhibits esophageal carcinogenesis by targeting the AKT/mTOR/p70S6 K and MAPK pathways. *Mol Carcinog*. 2018. doi:10.1002/mc. 22876.
28. Min W, Lin Y, Tang S, et al. AIP1 recruits phosphatase PP2A to ASK1 in tumor necrosis factor-induced ASK1-JNK activation. *Circ Res*. 2008;102:840-848.
29. Dale Rein I, Solberg Landsverk K, Micci F, et al. Replication-induced DNA damage after PARP inhibition causes G2 delay, and cell line-dependent apoptosis, necrosis and multinucleation. *Cell Cycle*. 2015;14(20):3248-3260.
30. Murad H, Alghamian Y, Aljapawe A, et al. Effects of ionizing radiation on the viability and proliferative behavior of the human glioblastoma T98G cell line. *BMC Res Notes*. 2018; 11(1):330.
31. Wang J, Zhu X, Hu J, et al. The positive feedback between Snail and DAB2IP regulates EMT, invasion and metastasis in colorectal cancer. *Oncotarget*. 2015;6(29):27427-27439.
32. Hidalgo M, Álvarez R, Gallego J, et al. Consensus guidelines for diagnosis, treatment and follow-up of patients with pancreatic cancer in Spain. *Clin Transl Oncol*. 2017;19(6):667-681.