Balanced Tiam1-Rac1 and RhoA Drives Proliferation and Invasion of Pancreatic Cancer Cells

Xingjun Guo1, Min Wang1, Jianxin Jiang2, Chengchen Xie1, Feng Peng1, Xu Li1, Rui Tian1, and Renyi Qin1

Abstract

Tiam1 is a rac1-specific guanine nucleotide exchange factor, and Tiam1-rac1 is involved in a number of cellular processes. Rac1 and RhoA act as molecular switches that cycle between GTP- and GDP-bound states to balance the activities of rac1 and RhoA. The downregulation of rac1 activity leads to upregulation of RhoA activity, which promotes invasion and migration of pancreatic cancers cells. At present, however, the role of Tiam1-rac1 and RhoA in pancreatic cancers is not fully understood. We found that Tiam1 was upregulated in pancreatic cancers and was significantly expressed in tumors without lymph node involvement or distant metastasis compared with cancers where there was involvement. Although Tiam1-rac1 signaling promoted pancreatic cancer cell proliferation and tumor growth via the Wnt signaling pathway in vitro and in vivo, inhibiting Tiam1-rac1 signaling did not prolong the overall survival time in vivo. This provided evidence that there was a balance between rac1 and RhoA activities in pancreatic cancers. Furthermore, only the combined inhibition of Tiam1-rac1 and RhoA had a beneficial effect on the growth of pancreatic cancers in vivo. Taken together, these results suggest that the progression of pancreatic tumors is partially controlled by the balance between Tiam1-rac1 and RhoA. Mol Cancer Res; 11(3): 230–9. © 2012 AACR.

Introduction

Pancreatic cancer is the fourth most common cause of cancer-related death worldwide and is always associated with a poor prognosis (1). Poor clinical outcomes may be due to most patients being diagnosed with advanced disease with extensive metastasis, high resistance of tumors to chemotherapy, and lack of effective chemotherapeutic agents (2). Investigation of the underlying mechanisms driving pancreatic cancer progression may reveal molecular targets for novel treatment strategies.

Tiam1 (T lymphoma invasion and metastasis 1) is a rac1-specific guanine nucleotide exchange factor and is believed to act as an oncogene in many cancers (3–6). Tiam1 maintains the specificity of rac1 toward specific downstream effector pathways, whereas rac1 regulates cell survival and cell-cycle progression (7, 8). Together, Tiam1-rac1 is a critical component in the biology of human tumors, in both transformed cells as and the cells in the tumor microenvironment (9–11). Tiam1 is a potent modifier of oncogenic Ras-induced skin tumor initiation, promotion, and progression. Furthermore, Tiam1 expression was found to be upregulated in a subset of carcinomas (12–14). In some other cancers, however, Tiam1 was found to suppress tumor invasion and metastasis (15, 16). As Tiam1 plays different roles depending on the cancer type, we investigated the role of Tiam1 in pancreatic cancer proliferation and invasion.

The mechanism underlying the cross-talk between Tiam1-rac1 and the canonical Wnt signaling pathway has been studied in colorectal cancer cells (17). Rac1 binds to the β-catenin/T-cell factor (TCF) transcription factor complex at Wnt-responsive promoters to enhance Wnt target gene transcription (18, 19). The canonical Wnt signaling pathway coordinates various cellular processes such as cell proliferation and cell fate determination during embryonic development and adult tissue maintenance (20). Furthermore, Wnt signaling has been shown to result in the stabilization and nuclear accumulation of β-catenin, the key mediator of the pathway. In the nucleus, β-catenin binds to members of the TCF/lymphoid enhancer factor (TCF/LEF) family of HMG box transcription factors to activate the transcription of target genes, including CDK4 and cyclin D1 (21).

In most situations, rac1 and RhoA exhibit an antagonistic relationship at multiple levels and there is balance between the activities of rac1 and RhoA (22–25). This opposition can be reciprocal and unidirectional, as observed in NIH3T3 cells, in which rac1 activation induces epithelial cell morphology, including cadherin-based junctions, accompanied by decreased RhoA activity. Elevation of RhoA activity reversed the phenotype, promoting a mesenchymal fibroblastic morphology (26). Quantitative in vivo fluorescence...
life-time imaging illustrated that RhoA is not only necessary for invasion but that subcellular spatial regulation of RhoA activity, as opposed to its global activity, is likely to govern invasion efficiency in vivo (27).

We hypothesized that Tiam1-rac1 and RhoA may be involved in pancreatic cancer proliferation and invasion. We tested this hypothesis and determined whether these factors may be potential therapeutic targets in patients with pancreatic cancer.

Materials and Methods

Cell lines and human tissues

Human pancreatic carcinoma cell lines (BxPC-3 and panc-1) were purchased from the Cell Repository, Chinese Academy of Sciences. BxPC-3 was grown in RPMI-1640 (Hyclone) and panc-1 was grown in Dulbecco’s Modified Eagle’s Media (DMEM; Hyclone), supplemented with 10% FBS (Gibco), 100 U/mL penicillin G, and 100 μg/mL streptomycin (Sigma-Aldrich), at 37°C in a humidified 5% CO2 incubator.

Surgical specimens of pancreatic cancers and corresponding normal pancreatic tissues were obtained from 53 patients, and surgical specimens of pancreatic benign tumors were obtained from 13 patients, all resected from January 2010 to December 2010. The 53 patients with pancreatic cancer consisted of 26 males and 27 females of mean age 57.3 years (range, 43–78 years). Histologically, all 53 cancers were adenocarcinomas. This study was approved by the Human Research Ethics Committees at the Tongji Hospital, Tongji Medical College, Huazhong University of Science and Technology (Wuhan, China). Written informed consent was obtained from all participants in this study.

Immunohistochemistry

Three-micrometer-thick paraffin-embedded sections were incubated overnight at 4°C with primary antibodies against Tiam1 (Santa Cruz; 1:100), cyclin D1 (Boster, China; 1:100), and ki67 (Boster; 1:100) and then were washed and incubated for 30 minutes with biotinylated goat anti-rabbit IgG. The sections were washed thoroughly, stained with diaminobenzidine, and examined by an Olympus BX41 microscope (Olympus). Six randomly selected fields of each section were examined by 2 pathology specialists blinded to patient diagnosis and outcome. Image-Pro Plus Version 6.0 (Media Cybernetics) software was used for analysis, and areas positive for Tiam1 expression and average optical density (AOD) were recorded.

Immunofluorescence

BxPC-3 cells grown on cover slips were incubated overnight at 4°C with primary antibody to E-cadherin (CST; 1:100), followed by incubation for 45 minutes at 37°C with CY3-conjugated goat anti-rabbit antibody (Boster). F-Actin distribution was detected using rhodamine phalloidin (Sigma-Aldrich) according to the manufacturer’s protocol. Slides were counterstained with 4’,6- diamidino-2-phenylindole (DAPI) to visualize the cell nuclei and photographed using a LEICA LCSLP2 confocal laser scanning microscope (×400) and analyzed by ZEN 2009 (Carl Zeiss).

shRNA silencing of Tiam1

The short hairpin RNA (shRNA) sequence targeting Tiam1 was cloned into the plasmid pLVX-GFP (Neuron Bio). As a control, scrambled shRNA with no target was cloned into pLVX-GFP. Lentiviruses were produced and titrated by standard protocols. Each preparation contained 1 × 10^8 TU/mL with an approximate 1:100 ratio of TU to physical particles, as quantitated by PCR. BxPC-3 and panc-1 cells were seeded in cell vessels the day before transduction, ensuring that the cells would grow to 30% to 40% confluence the next day and infected for 24 hours with lentivirus at an MOI of 50 in the presence of polybrene (5 mg/mL, Sigma-Aldrich). Stably transduced cells were obtained by sorting for GFP.

siRNA for rac1 and β-catenin

The specific nucleotide RNAs and scramble siRNA were chemically synthesized by Ribobio. The siRNA sequence targeting β-catenin corresponded to the coding region 5’- CAGTGTGTGGTTAGCTCTT-3’ and was generated from a siRNA duplex of the primers 5’-CAGUUGUG-GUUAACUCUCUdCdTdT-3’ (sense) and 3’-dTdTGU-CAACCAAUUUAGAA-5’ (antisense). The siRNA sequence targeting rac1 corresponded to the coding region 5’-TGAAGAGAGAGAGAGAAAdTdT-3’ (sense) and 3’-dTdTACUUCCU-CUCCCUUCUUU-5’ (antisense). BxPC-3 and panc-1 cells were transfected with rac1 and β-catenin specific siRNA using Lipofectamine 2000 (Invitrogen), according to the manufacturer’s instructions. The cells were cultured for an additional 48 hours to observe the effect of rac1 or β-catenin silencing on proliferation.

Western blot analysis

Polyvinylidene difluoride (PVDF) membranes containing electrophoretically separated proteins from human primary pancreatic cancer cells were incubated with rabbit anti-Tiam1 (Santa-Cruz; 1:500) or rabbit anti-β-catenin (CST; 1:1,000) antibody, followed by incubation with peroxidase-conjugated goat anti-rabbit IgG secondary antibody (CST; 1:2,000) and visualized by enhanced chemiluminescence (Boster).

RNA preparation and real-time PCR

Total RNA was extracted from human primary pancreatic cancer cells using a TRizol kit (Invitrogen) according to the manufacturer’s instructions. A 2 μg aliquot of each total RNA was reverse transcribed to cDNA using a ReverTra Ace qPCR RT Kit (Toyobo). Quantitative real-time PCR was carried out using a SYBR Green Realtime PCR Master Mix (Toyobo) according to the manufacturer’s instructions, and PCR primers were as follows: Tiam1, 5’-AAGAC- GTACTCAGGCCCATGTCG-3’ (sense) and 5’-GACC-
CAAATGTGCAGTC-3′ (antisense); cyclin-D1, 5′-CCGTGCCATCGGGAAGGATC-3′ (sense) and 5′-ATG-GCCAGCGGGAAGAC-3′ (antisense); CDK4, 5′-TC-TGGTACGAGCTCCC-3′ (sense) and 5′-GCCAACACTCCATGTCCA-3′ (antisense); c-jun, 5′-CCAGCAGTGCCGATAT-3′ (sense) and 5′-GGCAGTTAGAAGTGAA-3′ (antisense); c-myc, 5′-ATCACAGCCCTCTACTCAG-3′ (sense) and 5′-ACAGATTCCACC-AAGTTGC-3′ (antisense); and β-actin, 5′-TGCTCCTGGAGGCACT-3′ (sense) and 5′-GTCAGTAAAGCTGCTAGA-3′ (antisense).

Invasion and migration assays

Cell invasion and migration were analyzed using 24-well Corning Costar inserts of pore size 8 μm. For invasion assays, the upper surface of each insert was coated with diluted Matrigel (BD) for 6 hours in an incubator. Cells (100,000) were added to the upper chamber and incubated at 37°C, with migration assessed at 12 hours and invasion at 24 hours. Nonmigrating or noninvading cells were removed with a cotton swab from the top chamber. Cells remaining in the bottom chamber were fixed with 100% methanol, stained with 1% crystal violet in 2% ethanol, and quantified by MRI. The study was continued until day 100 to monitor growth progression/putative relapse of disease. Mice were assessed by MRI. The study was continued until day 100 to monitor growth was assessed on day 42 by palpation and confirmed microscopically. The remaining mice were kept alive for 24 hours and transfected for 24 hours. The medium was removed, fresh medium was added, and the cells were maintained in culture for an additional 48 hours. CCK-8 and EDU assays were conducted to assess growth inhibition.

G-LISA assay

RhoA and rac1 activity were measured using the G-LISA Assay System (Cytoskeleton) according to the manufacturer’s protocol.

Growth inhibition assay

Growth inhibition was measured using CCK-8 assays (Dojindo) and EDU tests (Ribobio). Briefly, cells were seeded in 6-well culture plates at a density of 50,000 cells per well or in 96-well culture plates at a density of 3000 cells/well grown for 24 hours and transfected for 24 hours. The medium was removed, fresh medium was added, and the cells were maintained in culture for an additional 48 hours. CCK-8 and EDU assays were conducted to assess growth inhibition.

In vivo treatment of established pancreatic cancers

Single-cell suspensions were either subcutaneously transplanted or orthotopically implanted into the pancreas of female BALB/c nude mice (HFK Bioscience). In the subcutaneous transplanted pancreatic cancer model, half the mice in each group were sacrificed 6 weeks after initiation of treatment, and the tumors were evaluated macroscopically and microscopically. The remaining mice were kept alive until day 80 to monitor the progression of disease. In the orthotopically implanted pancreatic cancer model, tumor growth was assessed on day 42 by palpation and confirmed by MRI. The study was continued until day 100 to monitor progression/putative relapse of disease. Mice were assessed with a 7-Tesla MRI system (Bruker Biospec) using a dedicated small animal coil. This study was conducted in strict accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of the Ministry of Health, PR. China. The protocol was approved by the Committee on the Ethics of Animal Experiments of Huazhong University of Science and Technology (Permit Number: 2011-S245). All surgery was conducted under sodium pentobarbital anesthesia, and all efforts were made to minimize suffering.

Statistical analyses

Results for continuous variables are presented as means ± SD unless stated otherwise. Treatment groups were compared with the independent sample t test. Pairwise multiple comparisons were conducted with the one-way ANOVA (two-sided). A value of P < 0.05 was considered statistically significant. All analyses were conducted using SPSS 17.0 (SPSS Inc.).

Results

Expression of Tiam1 in pancreatic cancer tissues

We first investigated whether Tiam1 protein was expressed in pancreatic cancers, pancreatic benign tumors, and normal pancreatic tissue. Immunohistochemical staining of tissue sections showed that normal pancreas tissue samples were negative for Tiam1, benign tumors were weakly positive, and adenocarcinomas were strongly positive (Fig. 1A). Most of the Tiam1 was localized in the cytoplasm (Fig. 1A). We found that Tiam1 expression differed significantly among the 3 sets of samples, normal pancreas, benign tumors, and adenocarcinomas (Fig. 1B), but there was no significant difference between the different grades of differentiation (Fig. 1C). Histologic examination of the 53 adenocarcinomas showed that 38 had lymph node involvement and/or distant metastasis (group A), whereas the other 15 could be categorized as without lymph node involvement or distant metastasis (group B). The expression of Tiam1 was significantly higher in tumors from patients without lymph node involvement and/or distant metastasis than in those with lymph node involvement and/or distant metastasis (Fig. 1D).

Tiam1-rac1 promotes proliferation and growth of pancreatic cancers via the Wnt signaling pathway

Our finding that Tiam1 expression was stronger in malignant tumors than in benign tumors and normal pancreas suggested that Tiam1 may play an important role in pancreatic cell proliferation and tumor growth. To assess the role of Tiam1 in pancreatic cancer biology, we generated stable BxPC-3 and panc-1 cell lines expressing an shRNA against Tiam1 using a lentiviral delivery system (Supplementary Fig. S1A). Western blot and real-time reverse transcription (RT)-PCR analyses showed that specific shRNA suppressed the expression of Tiam1 RNA and protein (Supplementary Fig. S1B and S1C). Moreover, the level of activated rac1 was downregulated in both cell lines (Supplementary Fig. S1D). We also found that cell growth and proliferation were markedly suppressed (Fig. 2A and B).

To elucidate the mechanisms involved in the growth inhibition induced by Tiam1 knockdown, we analyzed the
expression in these cells of mRNA encoding cyclin D1 and CDK4, genes that contribute to cell-cycle progression. Knockdown of Tiam1 downregulated the expression of cyclin D1 but not of CDK4 (Fig. 2C). As cyclin D1 is a target gene of the Wnt signaling pathway, we measured the mRNA expression of its main targets: c-myc, c-jun, and cyclin D1. We found that suppression of Tiam1 markedly downregulated the expression of c-myc and cyclin-D1.

Figure 1. Assay of Tiam1 protein expression in primary pancreatic tissue samples. A, immunohistochemical analyses of Tiam1 expression in normal pancreatic tissue (a and e) and in well (b and f), moderately (c and g), and poorly differentiated pancreatic cancers (d and h). Original magnifications: a–d, ×100; e–h, ×200. On the basis of Image-Pro Plus Version 6.0 software analysis, areas positive for Tiam1 expression and average optical density (AOD) were recorded. Every sample was randomly analyzed from three 100× fields of vision. Then, we used the values of the AOD as an indication of the relative quantity of Tiam1 and quantitatively analyzed Tiam1 expression in the following 3 situations. B, quantitative analysis of Tiam1 expression in normal pancreatic tissue, benign pancreatic tumors, and pancreatic cancers. Tiam1 expression was greater in pancreatic cancers than in benign pancreatic tumors (**, P < 0.01) and normal pancreas tissues (*, P < 0.01). C, quantitative analysis of Tiam1 expression in pancreatic cancers sorted by grades of differentiation. D, quantitative analysis of Tiam1 expression in tumors from patients without (group B) and with (group A) invasion and/or metastasis. Tiam1 expression was significantly stronger in group B than in group A (**, P < 0.05).
mRNAs while slightly decreasing the expression of c-jun mRNA (Fig. 2D). These findings suggested that Tiam1-rac1 promotes pancreatic cancer proliferation and tumor growth via the Wnt signaling pathway.

To explore the mechanism underlying the interaction of Tiam1-rac1 with the canonical Wnt signaling pathway, we conducted Western blotting of the expression of β-catenin in the cytosolic and nuclear fractions of BxPC-3 cells with or without Tiam1 knockdown. We found that Tiam1 knockdown did not affect β-catenin stabilization in the cytosol but markedly reduced β-catenin accumulation in the nucleus (Fig. 2E). This result was supported by our immunofluorescence experiments (Fig. 2F), indicating that Tiam1 is required for nuclear localization of β-catenin.

We next investigated the molecular mechanism by which Tiam1 is required for the nuclear localization of β-catenin. Tiam1 is a guanine nucleotide exchange factor that selectively activates the Rho-like GTPase rac1. We assayed β-catenin expression by Western blotting in the nucleus of BxPC-3 cells after transfection with si-rac1 or si-β-catenin or stimulation with the rac1-specific inhibitor NSC23766. NSC23766 and si-rac1 could decrease β-catenin expression in the nucleus and si-β-catenin could decrease rac1 expression in nucleus. Loading control is β-actin for cytoplasm and Lamin B, F, immunofluorescence confocal microscopy showing that Lamin B F, Western blotting of β-catenin expression in cytosolic and nuclear fractions of BxPC-3 cells. Tiam1 knockdown did not affect β-catenin expression in the cytosol but markedly reduced β-catenin expression in the nucleus. Loading control for cytoplasm is β-actin and for nucleus is Lamin B. F, immunofluorescence confocal microscopy showing that Lamin B F, Western blotting of β-catenin expression in cytosolic and nuclear fractions of BxPC-3 cells. Tiam1 knockdown did not affect β-catenin expression in the cytosol but markedly reduced β-catenin expression in the nucleus. Loading control for cytoplasm is β-actin and for nucleus is Lamin B.

**Figure 2.** Tiam1-rac1 promotes pancreatic cancer proliferation and tumor growth via the Wnt signaling pathway. A, EDU analysis of cell proliferation after Tiam1 knockdown. Tiam1 knockdown downregulates pancreatic cancer proliferation. BC, blank control or no treatment control; NC, nontargeting control. B, CCK-8 analysis of cell proliferation after Tiam1 knockdown. *P < 0.05 compared with control groups. C, real-time RT-PCR analysis of CDK and cyclin D1 expression after Tiam1 knockdown. **, P < 0.01 compared with the control groups. D, real-time RT-PCR analysis of the expression of Wnt signaling pathway target genes after Tiam1 knockdown. ***, P < 0.005; ****, P < 0.01 compared with control groups. E, Western blotting of β-catenin expression in cytosolic and nuclear fractions of BxPC-3 cells. Tiam1 knockdown did not affect β-catenin expression in the cytosol but markedly reduced β-catenin expression in the nucleus. Loading control for cytoplasm is β-actin and for nucleus is Lamin B. F, immunofluorescence confocal microscopy showing that Lamin B F, Western blotting of β-catenin expression in cytosolic and nuclear fractions of BxPC-3 cells after transfection with si-rac1 or si-β-catenin or stimulation with the rac1-specific inhibitor NSC23766. NSC23766 and si-rac1 could decrease β-catenin expression in the nucleus and si-β-catenin could decrease rac1 expression in nucleus. Loading control is β-actin for cytoplasm and Lamin B, H, for nucleus, real-time RT-PCR analysis of cyclin D1 expression after transfection with si-rac1 or si-β-catenin or stimulation with NSC23766. ***, P < 0.01 compared with control.
findings, we examined the role of Tiam1-rac1 in the regulation of cyclin D1 expression and found that rac1 knockdown or a rac1-specific inhibitor reduced cyclin D1 expression by about 50% (Fig. 2H). Using CCK-8 and EDU assays, we found that disruption of rac1 activity by transfection with si-rac1 or treatment with NSC23766 inhibited cell proliferation, similar to that observed with Tiam1 knockdown. Taken together, our findings showed that Tiam1-rac1 amplifies the Wnt signaling pathway activity by promoting β-catenin accumulation in the nucleus and augments Wnt target gene transcription.

**Tiam1-rac1 promotes pancreatic cancer growth in vivo**

To determine whether Tiam1-rac1 inhibition affects tumor growth in vivo, we tested the effects of Tiam1 knockdown and the rac1 inhibitor NSC23766 on human pancreatic cancers established in BALB/c nude mice. The details of the experimental setup are depicted in Fig. 3A. We found that both Tiam1 knockdown and NSC23766 significantly inhibited tumor growth (Fig. 3B and C). Histologic evaluation of the tumors confirmed that Tiam1 knockdown markedly reduced Tiam1 expression and somewhat reduced cyclin D1 and ki67 expression, with the latter also observed in cells treated with NSC23766 (Fig. 3E). During long-term follow-up, however, blocking Tiam1-rac1 did not prolong overall survival time (Fig. 3D).

**Balance between rac1 and RhoA activities in pancreatic cancer cells**

Because the expression of Tiam1 was significantly higher in the primary tumors of patients without lymph node involvement than with involvement and/or distant metastasis, we hypothesized that blocking Tiam1 may activate other signaling pathways and that these latter pathways may promote the invasion and metastasis of pancreatic cancer cells. A balance of rac1 and RhoA activities has been shown to regulate cell migration and invasion in many cancers, with downregulation of rac1 leading to activation of RhoA. We therefore hypothesized that Tiam1 knockdown would downregulate rac1, leading to the activation of RhoA, which promotes the invasion and metastasis of pancreatic cancer cells. Using G-LISA assays to measure Rac1 and RhoA activity after Tiam1 knockdown, we found that rac1 activity was downregulated, whereas RhoA activity was upregulated (Fig. 4A). We therefore examined rac1 and RhoA activity following treatment of BxPC-3 cells with NSC23766, C3 transferase, which nonselectively inactivates the GTPases RhoA, RhoB, and RhoC in vivo and in vitro, or Y27632, which is a selective inhibitor of Rho-associated protein kinases that has no effect on RhoA activity but can inhibit the effects of RhoA in vitro and in vivo. We found that NSC23766 downregulated rac1 and upregulated RhoA activity, whereas C3 transferase downregulated RhoA and upregulated rac1 activity (Fig. 4A). The combined use of NSC23766 and C3 transferase could downregulate both rac1 and RhoA activity (Fig. 4A). Y27632 did not impact RhoA activity but slightly upregulated rac1 activity (Fig. 4A). These findings indicate that inhibiting Tiam1-rac1 with Tiam1 knockdown or NSC23766 activates RhoA whereas inhibiting RhoA activity with C3 transferase activates rac1. Although Y27632 had no impact on RhoA activity, it can block its biologic effects and lead to the upregulation of rac1 activity. Thus, taken together, these findings show a balance between rac1 and RhoA activities in pancreatic cancer cells. As C3 transferase nonselectively inactivates the GTPases RhoA, RhoB, and RhoC, whereas Y27632 can selectively inhibit Rho-associated protein kinases and thus block RhoA biologic effects, we choose Y27632 for the next experiment.

**Combined inhibition of rac1 and RhoA downregulates pancreatic cancer cell invasion and migration**

We next investigated the role of Tiam1 in the invasive capacity of pancreatic cancer cells. In a wound-healing assay, we found that Tiam1 knockdown accelerated the wound closure rate at 24 hours compared with controls (Fig. 4B, Supplementary Fig. S2A). In a Transwell migration and invasion assay, knockdown of Tiam1 resulted in a 2-fold increase in the number of cells that passed through the membrane compared with the controls (Fig. 4C and 4D, Supplementary Fig. S2B and S2C). As expected, knockdown of Tiam1 was accompanied by enhanced invasiveness of BxPC-3 and Panc-1 cells. In the absence of knockdown, BxPC-3 cells had an epithelial-like morphology, with E-cadherin and F-actin arranged in a cortical pattern at cell–cell junctions. After Tiam1 knockdown, E-cadherin was no longer observed at cell–cell junctions, and F-actin stress fibers were present (Fig. 4E).

We next investigated the effects of rac1 and RhoA on the invasive capacity of pancreatic cancer cells. We found that treatment with the rac1-specific inhibitor NSC23766 markedly increased the percentage of migrated cells, whereas treatment with Y27632 had no effect (Figure 4F). In contrast, treatment with both NSC23766 and Y27632 decreased the percentage of migrated cells. Furthermore, Y27632 treatment completely blocked the enhanced invasiveness of BxPC-3 cells caused by Tiam1 knockdown, which had high RhoA activity (Fig. 4F). These results indicate that Tiam1 knockdown enhanced RhoA activity and upregulated invasive capacity. The combined inhibition of rac1 and RhoA diminished the migration and invasion of pancreatic cancer cells. Moreover, knockdown of Tiam1 or treatment with the rac1-specific inhibitor NSC23766 downregulated the proliferation of pancreatic cancer cells. Taken together, these findings indicate that the combined inhibition of rac1 and RhoA may decrease the proliferation and invasive activities of pancreatic cancer cells.

**Combined inhibition of rac1 and RhoA may have therapeutic benefits in pancreatic cancer**

On the basis of these promising findings, we assessed whether inhibition of rac1 and RhoA could increase progression-free survival in BALB/c nude mice with pre-established pancreatic cancers. Xenografts were established by orthotopic implantation of BxPC-3 cells into BALB/c nude mice and treatment was started 1 week after injection (Fig. 5A). Because only the combination of NSC23766 and Y27632 decreased
cell growth and invasion in vitro, we focused on this treatment regimen. Tumor growth was assessed on day 42 by palpation and confirmed by MRI (Fig. 5B). No tumors were detected in mice receiving combination therapy, so the study was continued until day 100 to monitor progression-free of disease. Over time, control animals bore large, life-limiting tumors and succumbed within 60 days of tumor implantation. All animals treated with NSC23766 alone showed progressive disease and succumbed within 60 days. Mice treated with NSC23766 and Y27632 showed significantly better long-term survival than those treated with NSC23766 alone, with 80% of the former group surviving to day 100 (Fig. 5C), and histologic evaluation of the liver showing the absence of metastatic sites (Fig. 5D).

Discussion

Patients with pancreatic cancer have a devastating prognosis, in part, due to the lack of effective chemotherapeutic agents (28). Tiam1 and its downstream target gene rac1 are frequently deregulated in human cancers. Tiam1-rac1 can antagonize RhoA activity directly at the GTPase level, and vice versa (24, 29). We provide in vitro and in vivo evidence that the balance of Tiam1-rac1 and RhoA drives the
proliferation and invasion of pancreatic cancer cells and provides a target for combined therapy.

We found that the expression of Tiam1 protein was significantly higher in pancreatic cancers than in corresponding normal pancreas cells and pancreatic benign tumors. Tiam1, a Rac1-specific GEF, has been associated with a variety of tumor types (30, 31), suggesting that Tiam1 may be important in the proliferation and growth of these cancers and act as a tumor promoter. We also found that the presence of lymph nodes or distant metastases was significantly associated with reduced expression of Tiam1. In fact, loss of Tiam1 expression has been associated with increased malignancy and invasiveness in skin tumor models, and Tiam1 protein expression was shown to be weaker during breast cancer (32) and gastric cancer progression (33), suggesting that Tiam1 may act as a suppressor of invasion and migration. Our immunohistochemical results suggested that Tiam1 may play opposite roles in pancreatic cancer cell proliferation and invasion. Thus, inhibiting Tiam1 activity in pancreatic cancer may not be beneficial.

We found that Tiam1 silencing in BxPC-3 and pANC-1 cell lines significantly arrested cell proliferation. The specificity of Tiam1-rac1 is determined by the effect of rac1 on specific downstream pathways. We proved that activation of rac1 promoted β-catenin accumulation in the nucleus and synergized with β-catenin to augment Wnt signaling–associated target genes transcription. Tiam1 was recently shown to be a Wnt-responsive gene and promote the formation and progression of intestinal tumors and breast cancers (19, 34, 35). Rac1 is a component of transcriptionally activating β-catenin/TCF complexes at Wnt-responsive promoters, and in the presence of Tiam1 the rac1 complex enhances target gene transcription (19). Moreover, β-catenin is a key mediator of the canonical Wnt pathway, as it is associated with members of the TCF family at Wnt-responsive promoters to drive the transcription of Wnt target genes (21). We found that Tiam1-rac1 amplified the Wnt signaling pathway activity by promoting β-catenin accumulation in the nucleus and that it augmented Wnt signaling pathway targeted gene transcription in pancreatic cancer cells.
In a pancreatic cancer subcutaneously transplanted model, we found that blocking Tiam1-rac1 slowed tumor growth but did not prolong overall survival time. Because Tiam1 expression was stronger in pancreatic tumors of patients without lymph node involvement and/or distant metastases than those with lymph node involvement and/or distant metastases, we hypothesized that blocking Tiam1-rac1 may activate other signaling pathways, which may promote the invasion and metastasis of pancreatic cancer cells.

The balance of rac1 and RhoA activities has been found to regulate cell proliferation and the invasion of many cancers (36). Downregulation of rac1 activity can activate RhoA through p120-catenin and p190RhoGAP (24) or through other signaling pathways. In contrast, downregulation of RhoA could activate rac1 through complicated mechanisms (22).

Thus, blocking Tiam1-rac1 by Tiam1 knockdown or use of the rac1-specific inhibitor NSC23766 leads to the upregulation of RhoA activity. RhoA promotes the invasion and migration of pancreatic cancer cells (27, 37, 38). We observed a balance between rac1 and RhoA activities in pancreatic cancer, finding that blocking Tiam1-rac1, either by Tiam1 knockdown or the rac1-specific inhibitor NSC23766, upregulated RhoA activity and promoted the invasion and migration of pancreatic cancer cells. We therefore hypothesized that only the combined inhibition of Tiam1-rac1 and RhoA would have a therapeutic effect on pancreatic cancers in vivo. In a pancreatic cancer orthotopically transplanted model, we found that the rac1-specific inhibitor NSC23766 inhibited tumor growth but did not prolong overall survival. In contrast, the combination of NSC23766 and the RhoA inhibitor Y27632 suppressed tumor growth and significantly enhanced long-term survival.

In conclusion, we have shown for the first time that a balance between the activities of rac1 and RhoA exists in pancreatic cancer cells. Tiam1-rac1 promotes pancreatic cancer cell proliferation via the Wnt signaling pathway. Although blocking Tiam1-rac1 slowed tumor growth, it had no benefit on overall survival as blocking Tiam1-rac1 activated RhoA, promoting the invasion and metastasis of pancreatic cancer cells. Taken together, we suggested that the progression of pancreatic tumors is partially controlled by the balance between Tiam1-rac1 and RhoA.

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

Authors' Contributions
Conception and design: X. Guo, R. Qin
Development of methodology: X. Guo, M. Wang, R. Tian, R. Qin
Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): X. Guo, M. Wang, J. Jiang, C. Xie, F. Peng
Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): X. Guo, M. Wang, X. Li, R. Qin
Writing, review, and/or revision of the manuscript: X. Guo, M. Wang, R. Tian
Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): J. Jiang
Study supervision: R. Tian, R. Qin

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Xingjun Guo, Min Wang, Jianxin Jiang, et al.


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